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PURIFICATION, PROPERTIES, AND AN ASSOCIATED TEMPLATE ACTIVITY OF *ESCHERICHIA COLI* S FACTOR

by

(C) MARION BELLE COULTER

A THESIS

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled, "Purification, Properties, and an Associated Template Activity of Escherichia Coli S Factor", submitted by Marion Belle Coulter in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Biochemistry.

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ABSTRACT

The *in vitro* copying of natural DNAs and certain defined DNAs by *E. coli* DNA polymerase I results in the production of covalently linked complementary sequences (ie., rapidly renaturable). There is a protein, S, which reduces or prevents the accumulation of such sequences. Once formed, these sequences are perpetuated by subsequent copying by DNA polymerase even in the presence of S factor.

Poly[d(T-T-G)•d(C-A-A)] containing no covalently linked complementary sequences has been prepared from a polymer which did contain such sequences by a physical separation of linked from unlinked strands in a density gradient followed by annealing and copying of the unlinked strands. Several procedures are described for the detection of incorrect sequences in the defined DNAs. These sequences are a problem especially with poly[d(T-T-G)•d(C-A-A)].

S factor has been purified by a combination of autolysis, gel exclusion chromatography, and ion exchange chromatography. The purified protein is associated with a template for the production of poly[d(A-T)•d(A-T)]. The protein has a molecular weight of 9500-12,000.

S factor blocks the accumulation of covalently linked

complementary sequences during the DNA polymerase-mediated copying of poly[d(T-G)•d(C-A)] and poly[d(T-T-G)•d(C-A-A)]. It is partially effective during the *in vitro* copying of RFII DNA from the bacteriophage PM2. Several models are presented for the mechanism of production of covalently linked sequences in these DNAs and for the action of S factor.

Templates for the production of unusual DNAs have been found in extracts of *E. coli*. These templates are not detectable by normal means but they can be copied by DNA polymerase. A by-product of an RNA polymerase preparation, contains templates for polypyrimidine•polypurine DNAs. A template for poly[d(A-T)•d(A-T)] is associated with S factor. This template co-purifies with S factor and remains associated with it in a urea-LiCl-CsCl density gradient. The template is sensitive to nuclease but only after deproteinization. The possible significance of these templates is discussed.

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LIST OF ABBREVIATIONS

A260	absorbance at 260 nm
A280	absorbance at 280 nm
AMV	avian myeloblastosis virus
dA	deoxyadenosine
d(r)AMP	deoxy(ribo)adenosine 5'-monophosphate
d(r)ATP	deoxy(ribo)adenosine 5'-triphosphate
BrdU	bromodeoxyuridine
BrdUTP	bromodeoxyuridine 5'-triphosphate
clc	covalently linked complementary
cm	centimeter
cpm	counts per minute
d(r)C	deoxy(ribo)cytidine
d(r)CMP	deoxy(ribo)cytidine 5'-monophosphate
d(r)CTP	deoxy(ribo)cytidine 5'-triphosphate
DEAE-	diethylaminoethyl-
DNA	deoxyribonucleic acid
DNase I	bovine pancreatic deoxyribonuclease I
DS	double-stranded
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetate
g	gram
g	gravity
d(r)G	deoxy(ribo)guanosine
d(r)GMP	deoxy(ribo)guanosine 5'-monophosphate

LIST OF ABBREVIATIONS (continued)

d(r)GTP	deoxy(ribo)guanosine 5'-triphosphate
M	molar
mM	millimolar
ma	milliampere
mg	milligram
ml	millilitre
u	micron
ug	microgram
ul	microlitre
ng	nanogram
nm	nanometer
nmole	nanomole
d(r)NMP	deoxy(ribo)nucleoside 5'-monophosphate
d(r)NTP	deoxy(ribo)nucleoside 5'-triphosphate
RFII	replicative form with one single-stranded nick
rpm	revolutions per minute
RNA	ribonucleic acid
S	svedberg
S	strand separability (factor)
Sarkosyl	sodium dodecyl sarcosinate
SDS	sodium dodecyl sulfate
SS	single-stranded
SSC	0.15 M NaCl - 0.015 M sodium citrate
TCA	trichloroacetic acid

LIST OF ABBREVIATIONS (continued)

Tm	thermal transition temperature
Tris	tris(hydroxymethyl) aminomethane
tRNA	unfractionated transfer ribonucleic acid
T	thymidine
TMP	thymidine 5'-monophosphate
TTP	thymidine 5'-triphosphate
UV	ultraviolet
rU	uridine
rUTP	uridine 5'-triphosphate
v	volume
V	volts

All temperatures are in Centigrade degrees.

CHAPTER I

INTRODUCTION

I S Factor and Unusual Template Activities in *E. coli* Extracts

The existence of a protein called S factor which prevents the accumulation of covalently linked complementary (i.e. rapidly renaturable) DNA during *E. coli* DNA polymerase-mediated copying of poly[d(T-G)•d(C-A)] was first reported by Paetkau (1969). The purification of S factor and some of its properties were subsequently described (Flintoff and Paetkau, 1974). A modified procedure for the purification of the protein as well as a further study of its properties comprise a portion of this thesis. The preparation of non-clc poly[d(T-T-G)•d(C-A-A)] was originally intended to provide an alternate substrate for S factor. The crude fraction (DIII) containing S factor activity used during the copying of this polymer appeared to contain a template for DNA of unusual sequence. The presence of another unusual template activity in crude S factor fractions was initially reported by Flintoff and Paetkau (1974). The characterization of both of these template activities is described in this thesis. The two template

activities, which result in the DNA polymerase-mediated production of a dG-dC rich polypyrimidine•polypurine DNA in one case and poly[d(A-T)•d(A-T)] in the other, are interesting in light of their association with DNA metabolizing enzymes.

II The Structure of DNA

The structure of DNA postulated by Watson and Crick (1953) seemed likely to fulfil two major requirements of genetic material, i.e. to exert a specific influence on the cell and to self-duplicate. The specificity of expression was predicted to be a property of the ordered sequence of the deoxynucleotide base pairs. The complementary nature of the two strands suggested that the molecule would be able to duplicate itself. This would be accomplished by each strand serving as a template for the formation of a new complementary strand. In such a scheme, replication would proceed semi-conservatively and indeed this was shown to be the case by Meselson and Stahl (1958). The discovery of an enzyme, DNA polymerase, which would copy DNA *in vitro* (Lehman *et al.*, 1958) provided a possible mediator for the replication process.

III Structures of Replicating DNA

The circular replicating Escherichia coli chromosome seen by Cairns (1963) added complications to the replication scheme. This structure suggested that both strands were copied simultaneously at a replicating fork in a double-stranded intact circle. This presented the problem of copying two strands of opposite polarity at a single site. DNA polymerase synthesizes DNA by adding onto a 3'-hydroxyl terminus and no evidence has been found for synthesis in a 3' to 5' direction (Goulian, 1971). The solution to the problem would seem to lie in an examination of the fine structure of the growing fork.

A structure involving discontinuities in the strands in the region of the replicating fork seemed a likely solution. Several variations on this theme have been suggested (Okazaki et al., 1968a; Guild, 1968) and some of these are shown in Figure 1a. Model C, Guild's "knife and fork" model, requires the action of a specific nuclease at the fork. The nuclease action would generate a structure resembling B. Two predictions may be made from such models: there may be single-stranded areas in the region of the growing fork and there should be low molecular weight pieces of DNA in the growing fork.

Evidence for single-stranded areas has come largely from electron microscopy. Replicating bacteriophage lambda

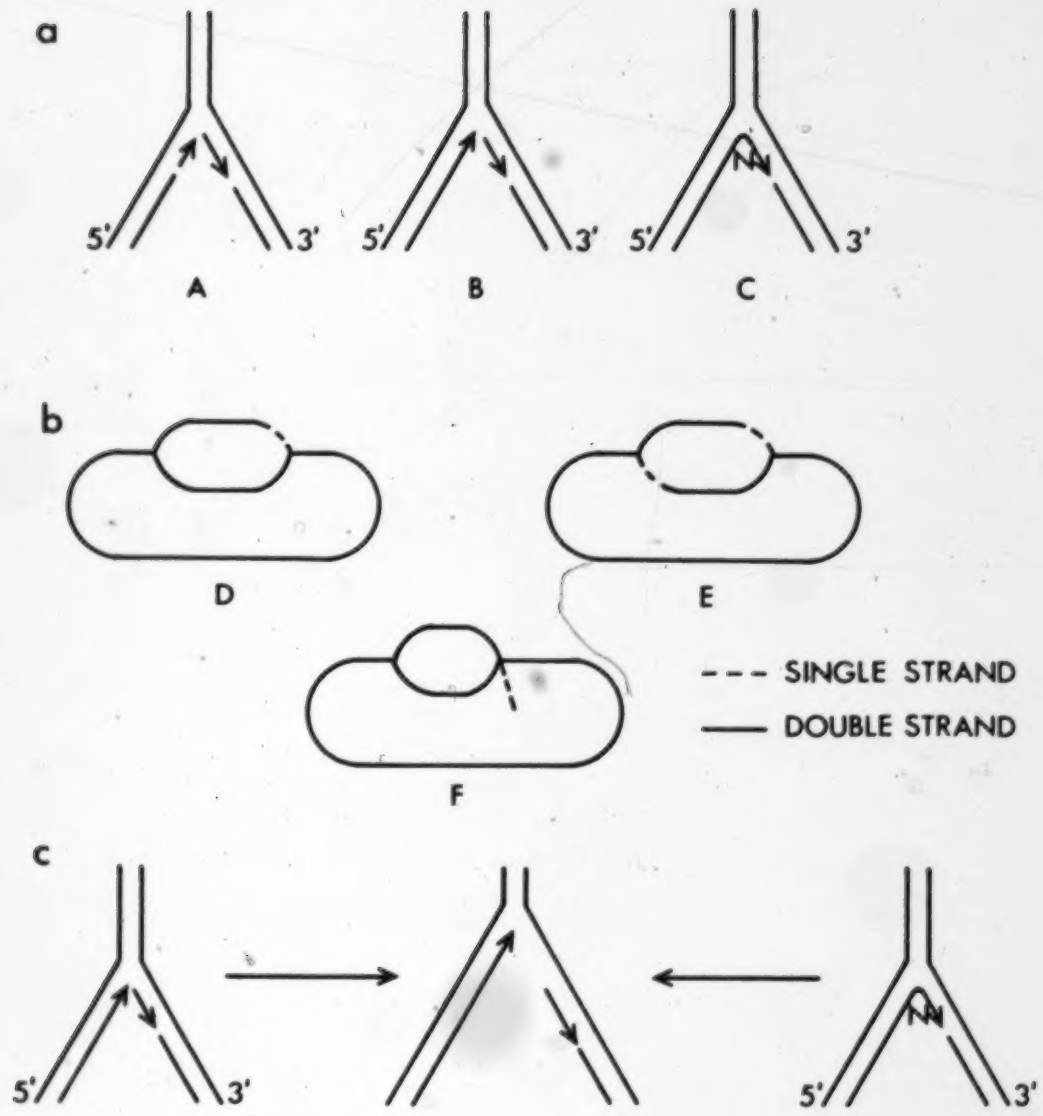


FIGURE 1. Structures of Replicating DNAs. (a) Models of the fine structure of the growing fork. (b) Single-stranded segments at the growing fork. (c) Origin of single-stranded segments. (N=site of nuclease action; horizontal arrows indicate a temporal sequence; other arrows indicate the direction of replication).

DNA has single-stranded regions at the growing points (Inman and Schnos, 1971). Lambda DNA is circular and replication proceeds in both directions around the circle at early times after induction. The most frequently found structures are shown in Figure 1b, structures D and E. Replicating bacteriophage T4 DNA has single-stranded regions (Delius et al., 1971) in the form of "whiskers" at the growing points (structure F, Figure 1b). These "whiskers" are thought to arise from structures such as D (Figure 1b) by a collapse of the parental single-stranded segment against its complement forcing out the newly-replicated strand. Replicating bacteriophage T7 DNA, a linear molecule, also showed single-stranded segments in the replicating fork (Wolfson and Dressler, 1972). Some common features of the single-stranded regions in these three DNAs are: they always occur on the daughter segment of the growing fork; and they are found in a trans arrangement (structure E, Figure 1b) if they are at more than one fork within a branch. These single-stranded segments could arise from structures such as B or C (Figure 1a) as shown in Figure 1c (Wolfson and Dressler, 1972).

The second prediction, that there should be low molecular weight pieces of DNA in the growing fork, has also been fulfilled. This has been demonstrated for E. coli and B. subtilus (Okazaki et al., 1968a), T4 (Sugino and Okazaki, 1972) and some mammalian cell DNAs (Painter and Schaeffer, 1969; Schandl and Taylor, 1969). The small DNA

pieces, "Okazaki pieces", are usually in the size range 8-11S and can be found by sedimentation of pulse-labelled DNA in either alkaline or neutral sucrose gradients. The small pieces are eventually incorporated into high molecular weight DNA (Okazaki et al., 1968a; Painter and Schaeffer, 1969; Schandl and Taylor, 1969). This last step may be performed by polynucleotide ligase (Okazaki et al., 1968).

There is a difference of opinion as to whether discontinuous replication occurs on one daughter arm of the replicating fork as in structures B or C (Figure 1a) or on both as in structure A (Figure 1a). The asymmetric arrangement of the single-stranded segments in T4, lambda and T7 supports models such as B or C. However, pulse-labelling experiments, in T4 for instance (Sugino and Okazaki, 1972) show that all the labelled material was of low molecular weight. Low molecular weight DNA from replicating lambda or T4 DNA shows no asymmetry in its hybridization to separated strands (Ginsberg and Hurwitz, 1970). Guild's "knife and fork" model (C, Figure 1a) can accommodate symmetric production of "Okazaki pieces" if it is combined with a structure like A (Ginsberg and Hurwitz, 1970).

IV Initiation of DNA synthesis

Assuming that the discontinuous model for replication is correct, then the initiation of the low molecular weight fragments must be accounted for. All known DNA polymerases require a template and a primer with a 3'-hydroxyl group (Kornberg, 1969; Kornberg and Gefter, 1972; Goulian, 1971). It has been suggested that specific oligonucleotide fragments may function as primers (Goulian, 1968) by hydrogen bonding to the template in short homologous regions. Short oligonucleotide fragments have been found in E. coli (Goulian, 1968; Schandl, 1972) and in mammalian cells (Schandl and Taylor, 1971). These fragments, varying from 8 to 12 nucleotides in length are much smaller than "Okazaki pieces".

DNA polymerase cannot initiate new strands de novo (Richardson, 1969) but RNA polymerase can. Several DNA polymerases will utilize a DNA template and an RNA primer (Wells et al., 1972; Chang and Bollum, 1972). Early indications that RNA synthesis might be involved in initiation of DNA synthesis came from studies of the inhibition of new DNA synthesis by rifampicin, an antibiotic which blocks RNA polymerase. This was observed for bacteriophage M13 (Brutlag et al., 1971) and E. coli (Lark, 1972). Through the use of temperature sensitive RNA polymerase mutants, Geider and Kornberg (1974) have recently shown that RNA polymerase is definitely involved in M13

replication and that it produces an RNA primer in a specific region of the chromosome. "Okazaki pieces" from E. coli (Sugino et al. , 1972; Hirose et al. , 1973) and Ehrlich ascites tumour cells (Sato et al. , 1972) seem to have an RNA segment associated with them. In the in vitro systems, permeable E. coli cells (Sugino and Okazaki, 1973) and isolated nuclei containing replicating polyoma DNA (Magnusson et al. , 1973), there is an RNA segment covalently linked at the 5' end of the DNA fragments. If RNA segments were covalently linked to the DNA in vivo as well, they would presumably be excised rather than joined into high molecular weight DNA. The covalent linkage to DNA and therefore initiation function of RNA has not been unequivocally demonstrated in vivo .

V Polymerases Involved in DNA Replication

Although numerous proteins are involved in DNA replication, of central importance is DNA polymerase itself. In E. coli there are three known DNA polymerases designated I, II, and III in order of their discovery.

The properties of DNA polymerase I have been reviewed by Kornberg (1969) and will be briefly outlined here. The polymerase consists of a single polypeptide chain of molecular weight 109,000. It binds to DNA at single-stranded regions and to nicks and ends but not to double-stranded areas. All four deoxynucleoside triphosphates compete for a

single binding site. The polymerase performs several enzymatic functions among which are polymerization in a 5' to 3' direction, hydrolysis of DNA in a 3' to 5' direction and hydrolysis of DNA in a 5' to 3' direction. The polymerase has an absolute requirement for a template and a primer with a 3'-hydroxyl group. The templates are copied with a very low frequency of errors.

The availability of the amber polA mutants, deficient in DNA polymerase I (De Lucia and Cairns, 1969), facilitated the discovery of the two other polymerases, II (Knippers, 1970) and III (Kornberg and Gefter, 1971). Several features distinguishing polymerases II and III from I are shown in Table I in terms of inhibitors (Table Ia) and template requirements (Table Ib).

There has been much speculation as to the precise division of labour among the three polymerases during replication. Polymerase III is considered essential for replication since temperature sensitive mutations in the polymerase III gene are lethal at non-permissive temperature (Gefter et al., 1971). Amber mutants deficient in polymerase II (Hirota et al., 1972a) and polymerase I are altered in their ability to repair DNA (Masker et al., 1973; De Lucia and Cairns, 1969). It has been proposed that polymerase I is a repair enzyme and not essential for replication. Observations by Okazaki et al. (1971), and Tait and Smith (1974) suggest that polymerase I is involved in,

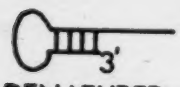
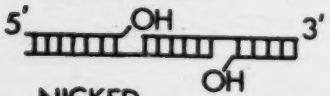
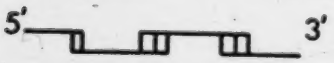
TABLE I

Distinguishing Features of Polymerases *in vitro*

a) Inhibition

Inhibitor	Polymerase Inhibited I	II	III	Reference
N-ethylmaleimide	-	+	+	1
Antiserum vs polymerase I	+	-	-	1
Ethanol (5%)	+	+	-	2
Inceased ionic strength	-	-	+	2

b) Template Requirements

Template	Used by Polymerase I	II	III	Reference
DOUBLE-STRANDED	-	-	-	2
 DENATURED	+	-	-	2
 NICKED	+	-	-	2
 GAPPED	+	+	+	2

References: 1. Kornberg and Gefter (1971).
2. Kornberg and Gefter (1972).

but not essential for, some early processing of low molecular weight DNA before its incorporation into high molecular weight material. It is perhaps significant that polA mutants contain residual polymerase I activity at 0.5 to 2% of the level found in wild type cells (Lehman and Chien, 1973); in fact recent unpublished genetic evidence from the same group has shown that polymerase I is essential in replication of the E. coli chromosome. Polymerase I is apparently indispensable for other functions. The maintenance of the colicinogenic factor E1 does not require polymerase III (Goebel, 1972) but it does require polymerase I in an apparently replicative function (Kingsbury and Helinski, 1973). Similarly, the bacteriocinogen Clo DF13 and the minicircular DNA of E. coli 15 require polymerase I but not polymerase III (Veltkamp and Nijkamp, 1973; Goebel and Schrempf, 1972).

A number of proteins in E. coli have been implicated in DNA replication by virtue of their mutation to temperature sensitive forms which prevent DNA synthesis at restrictive temperatures. These proteins are products of the dna loci. The mutants fall into two groups (Hirota et al., 1972b): those affecting initiation, dna A and dna C; and those affecting DNA elongation, dna B, D, E, F, and G. The dna E locus is the structural gene for polymerase III (Gefter et al., 1971). The dna F gene product is a ribonucleotide reductase (Fuchs et al., 1972). The dna G and dna C gene

products have been isolated (Wickner et al., 1973a,b) although their functions are unknown. The dna G and dna C products have molecular weights of 60,000 and 25,000 respectively. The dna C and dna D products co-purify and there is genetic evidence that the loci are the same (Wechsler, 1972).

VI Chemically Defined DNAs

The work described to this point has involved largely in vivo replication. In vitro studies also reveal many interesting facets of DNA synthesis. Useful templates for such studies have included isolated bacterial and bacteriophage DNAs as well as DNA polymers of defined sequence. The defined polymers were used extensively in the studies to be reported in this thesis. Therefore it is useful to consider their properties.

Chemically defined DNA polymers were initially designed as templates for transcription into polyribonucleotides of defined sequence and also as specific probes for the chemistry and enzymology of DNA (Khorana et al., 1965). The synthesis of specific oligodeoxynucleotides is accomplished by the sequential chemical condensation of nucleotides with appropriately protected groups followed by removal of the protecting groups and isolation of the oligomers (Ohtsuka et al., 1965). Some of the oligomers which have been used to make high molecular weight DNA polymers are listed in Table

II.

DNA polymerase I and the appropriate deoxynucleotide triphosphates can be used to copy these oligomers into high molecular weight polymers if three requirements are met:

a). Both members of a pair of complementary oligomers are present (Wells et al. , 1965; Wells et al. , 1967b). This demonstrates the requirement for a primer and a template.

b). The oligomers are at least a certain minimum length. For poly[d(A-T)•d(A-T)] (Kornberg et al. , 1964) and poly[d(T-G)•d(C-A)] (Wells et al. , 1965) the minimum size is an octanucleotide. The copying reaction has a lag period of 2 to 4 hours which is followed by rapid synthesis. The use of larger oligomers (10 or 12 residues) decreases the lag period to less than one hour. For repeating trinucleotide oligomers, the minimum size required is 8 to 12 residues (Wells et al. , 1967b) and for repeating tetranucleotide oligomers again 12 residues (Wells et al. , 1967a).

c). The complementary oligomers are able to form antiparallel Watson-Crick base pairs (Wells et al. , 1967b). For instance, with oligo[d(A-T-C)] plus oligo[d(A-T-G)] (of lengths 12 and 15 respectively) there is extensive synthesis of a polymer having strands of opposite polarity while oligo[d(A-T-C)] with oligo[d(T-A-G)] showed no reaction.

TABLE II

Defined Sequence Oligodeoxynucleotide Precursors to Polydeoxynucleotides

Length of Repeating Unit			
1	2	3	4
d(T) [2]	d(T-G) [3]	d(T-T-G) [5]	d(T-A-T-C) [7]
d(A)	d(C-A)	d(C-A-A)	d(T-A-G-A)
	d(T-C) [2]	d(T-T-C) [5]	d(T-T-A-C) [7]
	d(G-A)	d(G-A-A)	d(T-A-A-G)
	d(A-T) [4]	d(T-A-C) [5]	
		d(G-T-A)	
		d(A-T-C) [5]	
		d(G-A-T)	
		d(T-C-C) [6]	
		d(G-G-A)	

[1]Oligomers are shown in complementary pairs.
Oligomers were used for synthesis of polymers by:
[2]Byrd et al. (1974); [3]Wells et al. (1967b);
[4]Kornberg et al. (1964); [5]Wells et al. (19671);
[6]Morgan et al. (1974); [7]Wells et al. (1967a).

Nearest neighbour analyses and differential labelling of the strands (Wells et al. , 1965; Wells et al. , 1967a,b) have shown that both strands of the polymers are synthesized equally.

The copying of the various oligomers by DNA polymerase I produces high molecular weight polymers of the correct sequence. The polymers can be used as templates for subsequent copying although in some cases such as poly[d(T-T-G)•d(C-A-A)], poly[d(T-T-C)•d(G-A-A)], poly[d(T-C)•d(G-A)], and the repeating tetranucleotide polymers, there have been problems with the facility and/or fidelity of copying (Wells et al. , 1967a,b).

Two mechanisms have been considered for the production of high molecular weight polymers from oligomers. The first (Figure 2a) is a reiterative mechanism of synthesis-slippage-synthesis (Kornberg et al. , 1964). This mechanism was originally postulated for oligo[d(A-T)] but also applies to any oligomers with repeating units. A second mechanism (Figure 2b), the staggered mechanism, requires the association of complementary oligomers to form a double-stranded complex with single-stranded gaps (Burd and Wells, 1970). The gaps would be filled in by DNA polymerase or by alignment of the oligomers. The two mechanisms are not mutually exclusive since slippage obviously would play a role in the alignment of the oligomers for the second mechanism and may be involved in the extension of the

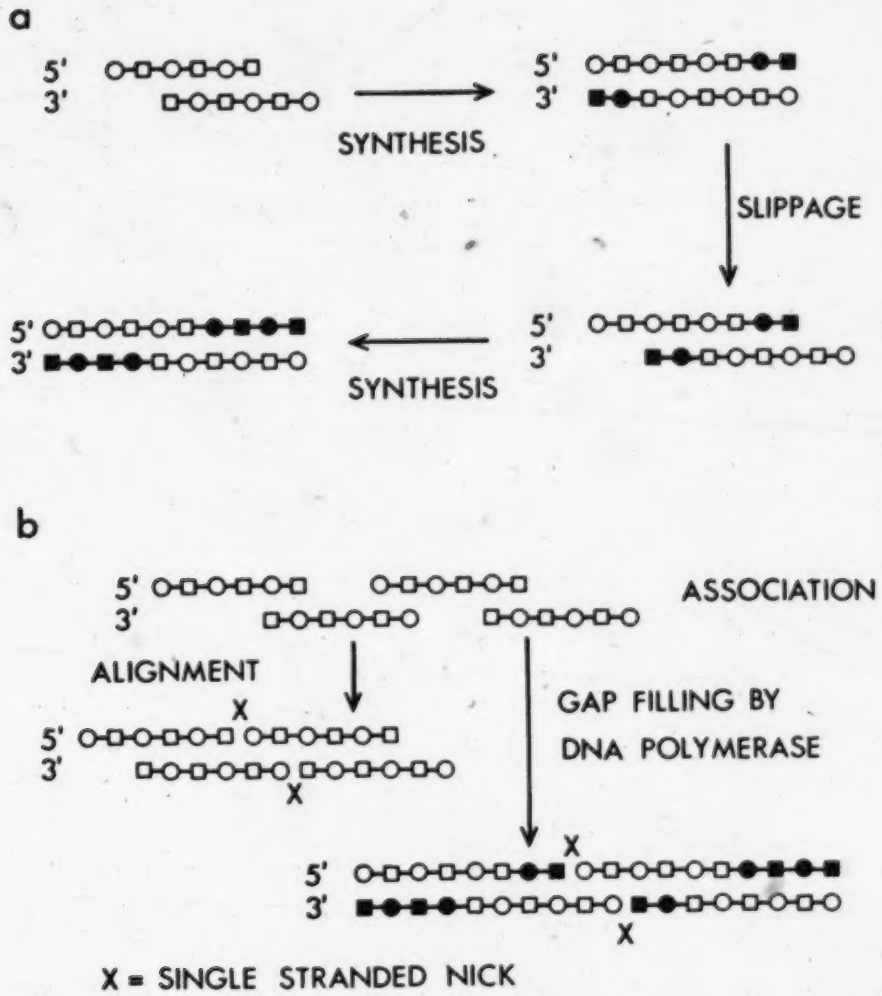


FIGURE 2. Mechanisms for Production of High molecular Weight Polymers from Oligomers. (a) reiterative mechanism; (b) staggered mechanism. Repeating units: (□-□) parental; (■-■) newly formed.

aligned complex.

The copying of high molecular weight template polymers is believed to proceed by two mechanisms: slippage (Figure 3a) and strand displacement (Figure 3b) (Coulter et al., 1974). Increasing the length of the repeating unit from a di- to a tri- to a tetranucleotide would make slippage less likely.

The polymers copied from the oligodeoxynucleotides are high molecular weight, double-stranded, DNA-like molecules (Wells et al., 1965). For example, the molecular weight of poly[d(T-G)•d(C-A)] can be as high as 10^6. Electron micrographs of poly[d(T-G)•d(C-A)] show rigid double-stranded molecules of length 0.5 microns. The physicochemical properties (Tm, extinction coefficients, buoyant densities, and CD spectra) of the high molecular weight polymers (Wells et al., 1970) showed variations from predictions based on natural DNAs and base composition. These variations are likely due to the regular sequence.

Polypyrimidine•polypurine DNAs differed from their mixed pyrimidine-purine sequence isomers in their template capacity, transcription rates, extinction coefficients, Tm's, buoyant densities, actinomycin D binding, ability to form 3-stranded structures and x-ray diffraction patterns (Wells et al., 1970). The mixed pyrimidine-purine isomers showed a closer resemblance to natural DNAs. The segregation

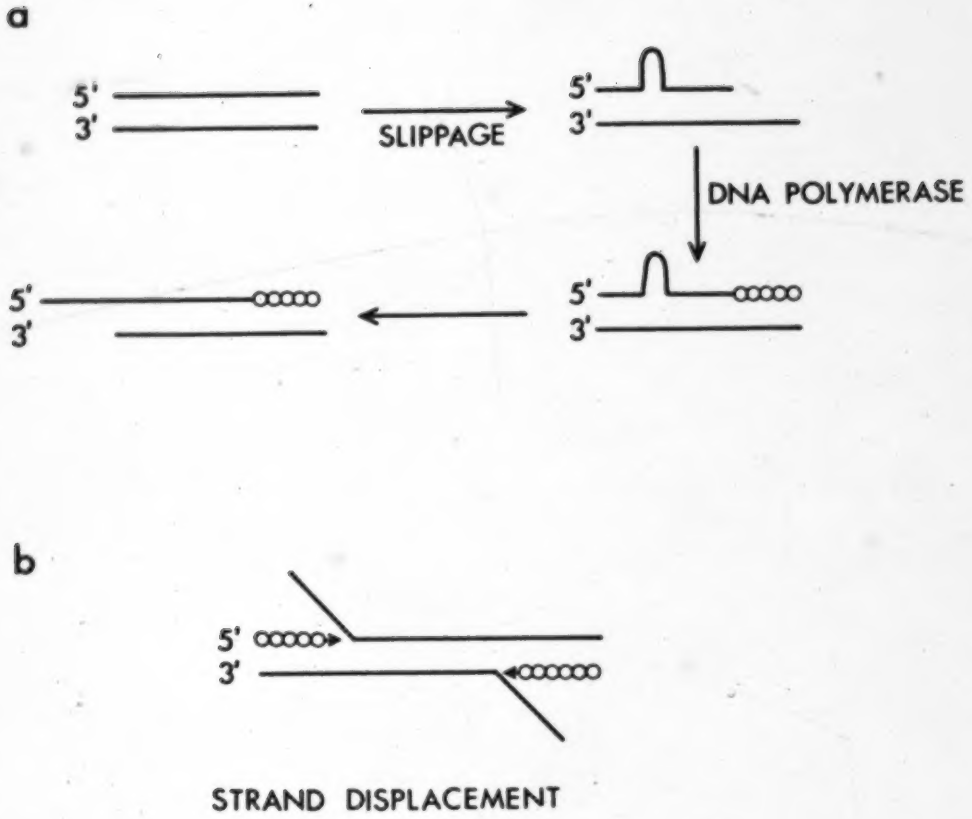


FIGURE 3. Slippage and Strand Displacement Models for Copying of High Molecular Weight DNA Polymers. (a) slippage mechanism; (b) strand displacement. (–) parental DNA; (ooo) newly formed DNA.

of the smaller pyrimidine and the larger purine bases into different strands in a polypyrimidine•polypurine DNA may cause a structural change from the normal double helix.

DNA polymers of defined sequence and the specific mRNAs transcribed from them have been put to many uses some of which have been reviewed by Khorana et al. (1966). They include transcription studies, amino acid incorporation and codon assignments using defined mRNAs, missense to sense suppression studies and translation studies. The DNA polymer poly[d(T-G)•d(C-A)] has been particularly convenient for studies of S factor activity and synthesis of covalently linked complementary DNA (Flintoff and Paetkau, 1974; Coulter et al., 1974).

VII DNA with Covalently Linked Complementary Sequences

Covalently linked complementary DNA is a peculiar structure which results in rapid renaturation of DNA. Normally, renaturation is a second order reaction limited by the rate of collision between complementary sequences (Wetmur and Davidson, 1968). However, a covalent link between complementary sequences provides a stable nucleation site permitting very rapid reassociation. Such structures are referred to as clc for "covalently linked complementary" (Morgan and Paetkau, 1972). There are several ways, both artificial and natural, that such structures can arise.

Cross-links between complementary DNA strands can be introduced chemically by bifunctional alkylating agents such as nitrous acid (Becker et al. , 1964) nitrogen mustards (Kohn et al. , 1966), or certain antibiotics such as mitomycin or porfiromycin (Iyer and Szybalski, 1964). Such cross-linked DNAs behave like double-stranded DNA after denaturation, i.e. they have a buoyant density in CsCl typical of double-stranded DNA and they retain transforming activity.

DNAs isolated from B. subtilus, E. coli , Haemophilus influenzae, and calf thymus appear to contain 5-6% renaturable DNA (Alberts and Doty, 1968). The renatured DNA is of double-stranded character. The stability of the linkage suggests that it is covalent. It has been suggested that the covalent link in this case is a result of shearing and is therefore probably an artefact of isolation (Alberts, 1968).

Terminal cross-linking of DNAs can be produced by various enzymatic means. Weiss (1970) found that T7 DNA could be made 40% renaturable by an ATP and magnesium-dependent enzyme system from T4-infected E. coli . The mechanism postulated to explain this is shown in Figure 4a. A similar mechanism has been found in T7-infected E. coli (Sadowski et al. , 1974). Copying of single-stranded T7 DNA by T4 DNA polymerase also produces a terminally cross-linked structure (Englund, 1971). A model for this process is shown

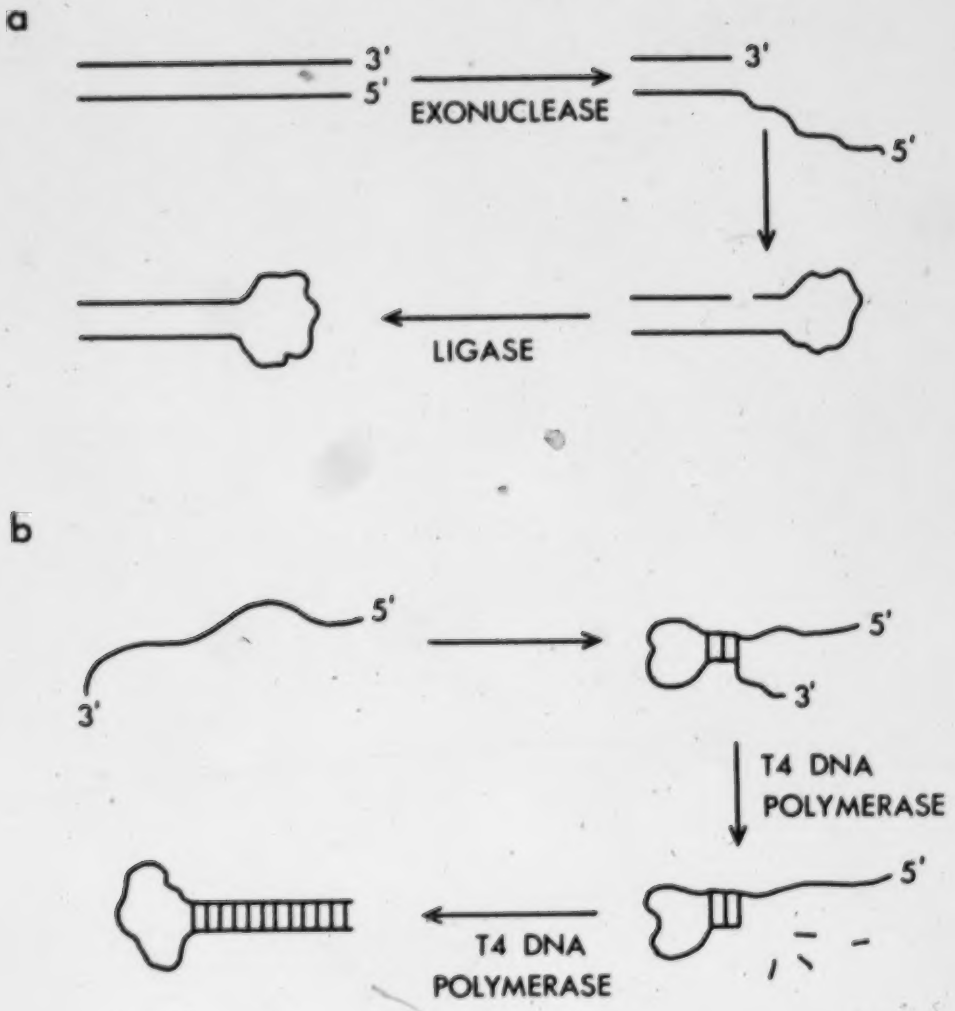


FIGURE 4. Mechanisms for Enzymatic Production of Terminally Cross-linked T7 DNA. (a) ligase-mediated; (b) T4 DNA polymerase-mediated.

in Figure 4b. Calf thymus DNA polymerase utilizes poly[d(A)] to give double-stranded structures with terminal cross-links (Hayes et al., 1971). The initial step involves a 3'-terminal addition of TMP residues followed by hydrogen bonding of these residues to the poly[d(A)] strand similar to the process seen in Figure 4b. The remainder of the poly[d(A)] strand is then copied. The terminal joining in all these cases is a single-stranded loop. Two DNA polymers may be joined at base-paired ends by T4 ligase (Sgaramella et al., 1970) and in this way may produce renaturable structures if the joined sections are complementary.

Clc structures may arise naturally as a consequence of intrastrand complementarity. The primary example of this is the inverted repetition or palindrome represented by the following simple model:

5'...A B C t C'B'A'...3'

3'...A'B'C't C B A ...5'

Upon renaturation such structures form "hairpin" duplexes with single-stranded loops represented by "t". The duplexes are recognizable by their retention on hydroxyapatite, their resistance to single strand-specific endonucleases, and electron microscopy. Palindromes have been found in DNA from HeLa, Xenopus, mouse, Drosophila, and Triturus cells (Wilson and Thomas, 1974). In these cases the "t" region appears to

be short. Resistance to single strand endonucleases suggested a length of one nucleotide. Similar structures have been seen in E. coli plasmid DNA (Sharp et al. , 1973). In this case, electron microscopy shows the "t" region to be quite large. The minimum size for a single-stranded loop in a hairpin structure is 2-4 nucleotide residues (Scheffler et al. , 1968; Neselson et al. , 1972). Some evidence of intrastrand complementarity has been seen for the single-stranded DNAs of bacteriophages M13 (Forscheit and Ray, 1970) and fd (Schaller et al. , 1969). These structures cannot be considered palindromic since the DNA is single-stranded.

Clc DNA may arise from the growing fork of replicating DNA. Guild's (1968) "knife and fork" model of replication predicts the occurrence, however transient, of a clc structure at the growing fork. There is evidence for such a structure in the apparently double-stranded character of pulse-labelled T7 DNA (Barzilai and Thomas, 1970). More conclusive evidence comes from a consideration of electron micrographs of replicating circular colicin E1 DNA (Fuke and Inselburg, 1972). Among the single-branched circles of the rolling circle type are some having supercoiling in the circular segment. This suggests a topological constraint to unwinding at the joining of the branch and the circle. A covalent link between the newly replicated strands at the growing fork would provide such a constraint.

The paucity of observations of clc structures arising from the growing fork *in vivo* is not convincing proof that these structures do not occur. The persistence of this sort of clc DNA beyond a transitory existence at replication would surely be lethal to the organism. Clc structures are best seen in DNAs that have been copied *in vitro* by DNA polymerase, where their occurrence suggests an incomplete reconstitution of the components of the replication mechanisms of the organism.

The products of native DNA templates copied *in vitro* by *E. coli* DNA polymerase I rapidly renature to double helical structures after heat or alkaline denaturation (Schildkraut *et al.*, 1964). Electron micrographs of these products show multi-branched structures (Inman *et al.*, 1965). Copying of the circular DNA of bacteriophage PM2 also produces a multi-branched, renaturable structure (Masamune and Richardson, 1971). Purified AMV polymerase and *Micrococcus luteus* polymerase produce clc DNA during *in vitro* synthesis (Leis and Hurwitz, 1972; Harwood and Wells, 1970).

The chemically defined DNA, poly[d(T-G)•d(C-A)] yields clc structures when copied by purified *M. luteus* or *E. coli* DNA polymerases (Harwood and Wells, 1970; Paetkau, 1969). This polymer is particularly well-suited to the detection of clc DNA since the segregation of ionizable bases into different strands permits the separation of non-covalently

linked strands in an alkaline CsCl density gradient. Clc poly[d(T-G)•d(C-A)] bands at an intermediate density.

A convenient assay for clc DNA combines the enhanced fluorescence of ethidium bromide when bound to double-stranded polynucleotides and the inability of DNAs to renature after heating and rapid cooling at very low salt (Morgan and Paetkau, 1972). (This assay is described fully in Chapter II). Figure 5 presents a summary of various clc DNAs and the structures expected following heat denaturation and rapid cooling.

VIII Unusual Sequences in DNA

The sequence of bases in DNA was predicted and found to be the source of the specificity of genetic expression through translation. However, there are sequences which may not be informational in the genetic sense but rather provide specific recognition sites for the binding of certain DNA metabolizing proteins. Some interactions of proteins with the chromosome occur with sufficient specificity that there are likely to be unique sequences involved. For instance, it has been shown by denaturation mapping that both lambda and P2 bacteriophage have unique locations for intiation of DNA synthesis (Schnos and Inman, 1970, 1971).

Other interactions are clearly linked to sequence. An integral part of the operon model of Jacob and Monod (1961)

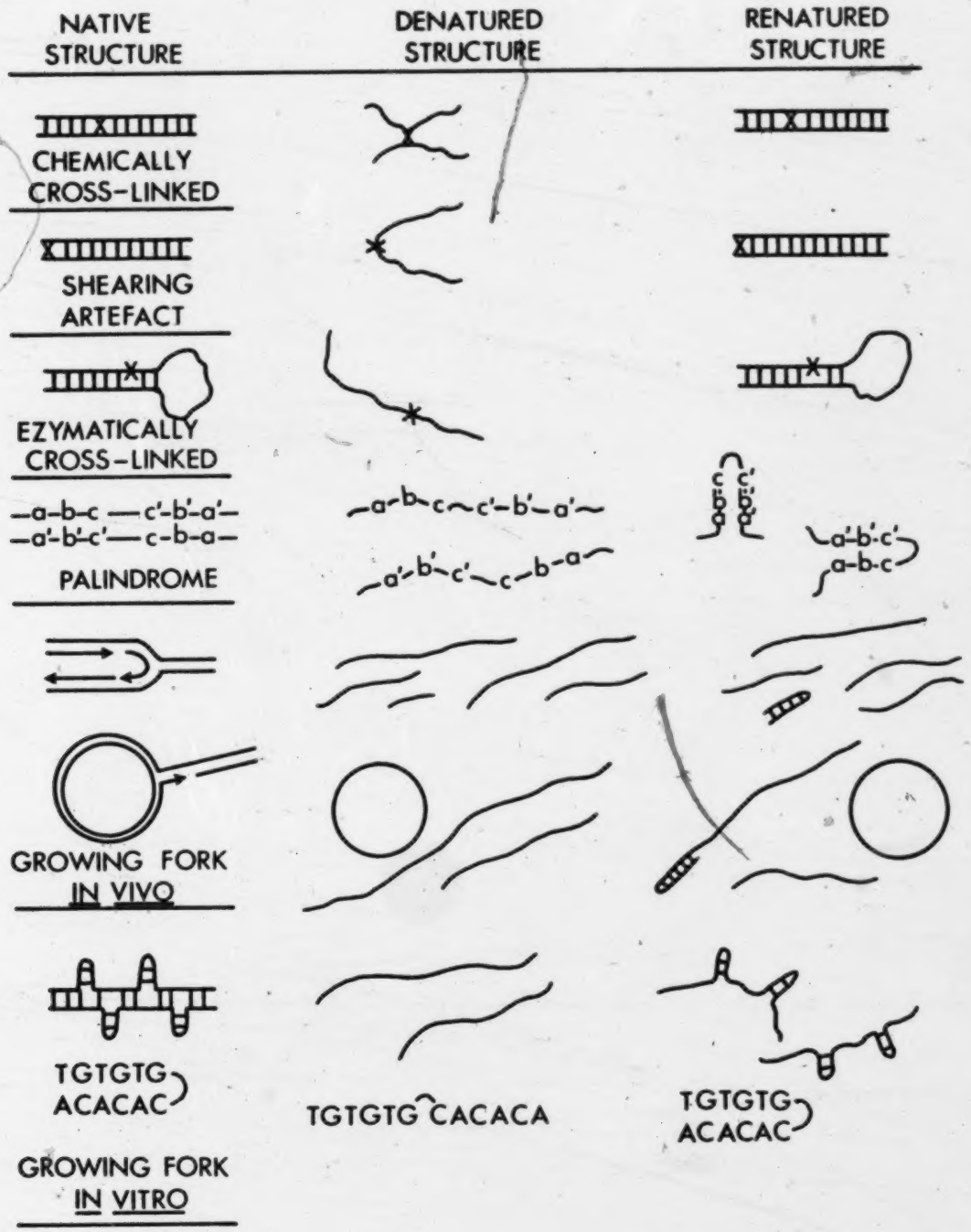


FIGURE 5. Clc DNAs and the Expected Results of Heat Denaturation and Cooling. (x=cross-link).

is the operator, which, through an interaction of its specific sequence with a repressor molecule controls the transcription of a particular structual gene. Jacob and Monod suggested that the repressor was an RNA with a sequence complementary to that of the operator. The repressor-operator systems of the E. coli lactose operon and the lambda prophage have been extensively studied. In both cases the repressors are polypeptides rather than RNAs (von Hippel and McGhee, 1972).

Lambda is maintained in a passive prophage state by blocking the transcription of essentially all lambda DNA except for the gene specifying the repressor (Szybalski et al. , 1969). The operator site can be isolated by binding with the repressor proteins (Pirrotta, 1973). There are at least four and perhaps up to twelve specific repressor recognition sites within the lambda operator (Maniatis et al. , 1973).

Transcription of the lactose operon is blocked by the lactose repressor unless an inducer is present. The lactose repressor binds well to dA-T rich DNAs and to poly[d(A-T)•d(A-T)] itself (Lin and Riggs, 1970). This suggested that the operator site might be dA-T rich. The lactose operator has been isolated (Gilbert et al. , 1973) in much the same way as used for the lambda operator. Its sequence has been determined indirectly through its RNA transcript (Gilbert et al. , 1973). The repressor binding site consists of 24 base

pairs of which 16 fit into a pattern with a twofold axis of symmetry as shown below (symmetry regions are bracketed):

T G G{A A T T G T}G{A}G{C}G{G}A{T}A{A C A A T T}
A C C{T T A A C A}C{T}C{G}C{C}T{A}T{T G T T A A}

Twelve of the 16 base pairs are dA-T pairs. Such a structure is similar to the palindromes described previously. The repressor protein, a tetramer, may recognize the twofold axis of symmetry.

Sequences having twofold axes of symmetry are also implicated as recognition sites for Type II restriction and modification enzymes recently reviewed by Boyer (1974). (Type I enzymes are complex and their specificities have not been elucidated). The two enzymes recognize the same substrate. The restriction enzyme, an endonuclease, cleaves two phosphodiester bonds in the sequence unless certain bases in the sequence have previously been methylated by the modification enzyme. The Type II restriction and modification enzymes are best known in H. influenzae (Kelly and Smith, 1970), and in E. coli carrying R factors (Boyer, 1974). It has been proposed (Kelly and Smith, 1970) that, as suggested for the lactose operator-repressor system, the enzymes may have a twofold axis of symmetry in their subunits permitting simultaneous action on both strands of the DNA.

The recognition sites for transcription by RNA polymerase also appear to be highly specific. There must be provision for initiation at certain DNA sites, for asymmetric transcription of strands and for termination of transcription. A correlation has been found between the number of poly[r(G)] binding sites on a DNA strand and the asymmetry of transcription. In the bacteriophage T7 only one strand is transcribed and this is the same strand which preferentially binds poly[r(G)] (Summers and Szybalski, 1968). The transcription of lambda DNA is asymmetric in a given region of the chromosome and the orientation of poly[r(G)] binding sites corresponds to the orientation of transcription in that region (Champoux and Hogness, 1972). On the other hand, the sites of RNA polymerase binding which have been examined in fd bacteriophage (Heyden et al., 1972) and lambda (LeTalaer and Jeanteur, 1971) appear to be slightly enriched for A-T sequences. Poly[d(A-T)•d(A-T)] is known to bind more RNA polymerase than does T7 DNA (Jones and Berg, 1966). As discussed earlier, polypyrimidine•polypurine DNA has an abnormal double helical form so that the segments of such DNA responsible for poly[r(G)] binding would be expected to cause local structural alterations in the DNA. It has been suggested (Szybalski et al., 1969) that such segments adjacent to a specific sequence may provide recognition sites for RNA polymerase. The presence of the structural singularity

introduced by the pyrimidine•purine segment would decrease the number of residues required for the sequence to be unique. A similar proposal (Champoux and Hogness, 1972) visualizes the pyrimidine•purine segments as markers for the division of mRNA. The pyrimidine•purine sites may also affect the asymmetry of the transcription. Evidence for this comes from the preferential transcription of the pyrimidine strand of defined polypyrimidine•polypurine DNAs (Morgan, 1970). It is unlikely that factors other than sequence are affecting the asymmetry in this case.

It can be calculated that in an E. coli chromosome comprised of approximately 3.5×10^6 base pairs, a sequence must be at least 12 base pairs long to be unique. Theoretically a protein has access to the determinant groups on the edges of the stacked bases through the grooves in the helix. However, the thermodynamic and steric demands placed on a protein to discriminate 12 positions in this way may be unrealistic (von Hippel and McGhee, 1972). Therefore other sequence features may be involved in a bipartite recognition process. A pyrimidine•purine segment (Szybalski et al., 1969) or a symmetric region may provide a coarse criterion for identification by the protein. Similarly, the altered secondary structure of dA-T rich DNA (Bram, 1971) may function as a specific marker for recognition. The sequence then involved for the fine recognition may be much shorter than 12 base pairs. Whatever the precise recognition

process, it seems likely that many proteins interact with DNA at specific sites by virtue of their affinity for a particular unusual sequence.

CHAPTER II

MATERIALS AND METHODS

I Materials

A. Chemicals

Unlabelled nucleotides were purchased from P-L Biochemicals; labelled nucleotides from New England Nuclear or Schwarz/Mann. The density-labelled nucleotide BrdUTP was prepared in the laboratory of Dr. A. R. Morgan using a modification (D. E. Pulleyblank and A. R. Morgan, unpublished) of the the procedure of Inman and Baldwin (1964). Ethidium bromide was obtained from Sigma; Ultrapure Ammonium Sulfate from Mann; phenol from McArthur Chemical Co.; urea from J. T. Baker Chemical Co.; CsCl from Pierce Chemical Co.; cesium sulfate from American Potash and Chemical Corp.; and Aquasol from New England Nuclear. The protein stain Coomassie Brilliant Blue was a product of Colab Laboratories. Other gel electrophoresis chemicals were purchased from Eastman Organic Chemicals.

Agarose 15M (200-400 mesh), the mixed bed resin AG501-X8(D), Dowex 50WX-4, and Biogel HT (hydroxyapatite) were obtained from Bio-Rad. Phosphocellulose (P11) and DEAE-

cellulose (DE23) were obtained from Whatman. The Sephadex resins, G-25 (20-30 u), G-50 (20-80 u), G-75 (40-120 u), G-100 (40-120 u), and DEAE-Sephadex A25 were purchased from Pharmacia Fine Chemicals. Blue Dextran 2000 was also obtained from Pharmacia.

All other chemicals were reagent grade.

B. Biological Materials

Pancreatic DNase I and snake venom phosphodiesterase I were obtained from Worthington Biochemicals Corp. Bacterial alkaline phosphatase, obtained from Worthington Biochemicals, was purified in the laboratory of Dr. A. R. Morgan by the method of Weiss et al. (1968). Pronase was purchased from Calbiochem and was pretreated before use by heating a 20 mg/ml solution at 80° for 10 minutes. E. coli endonuclease I was purified by Dr. W. F. Flintoff using the method of DeWaard and Lehman (1966). A unit of endonuclease I is defined as causing a hyperchromicity of 0.001 A260 at 25°. E. coli B cells, grown to 3/4 log phase in minimal medium, were obtained from the Grain Processing Co., Muscatine, Iowa. Proteins used as molecular weight markers were obtained from Pharmacia Fine Chemicals. R17 bacteriophage was a gift from Mrs. L. Frost.

C. Nucleic Acids

Calf thymus DNA was obtained from Worthington

Biochemicals; the decadeoxynucleotides oligo[d(T-G)] and oligo[d(C-A)] from Collaborative Research Inc. The chemically defined DNA polymers have been described (Wells et al., 1967). The following gifts are acknowledged: PM2 RFII DNA from Dr. B. Eskin; unfractionated yeast tRNA (Boehringer) from Dr. C. J. Smith; and S factor free of poly[d(A-T)•d(A-T)] from Dr. W. F. Flintoff.

II Methods

A. Preparation of Reagents

All buffers and solutions were millipore filtered through an HAWP 04700 membrane before use. Urea was purified by passing 10 M solutions over a column (3.2x5 cm) of the mixed bed resin AG501-X8(D). Phenol was freshly distilled and saturated with buffer before use. Cesium sulfate was recrystallized from hot water.

Unlabelled r- and dNTPs were dissolved in water and neutralized with free "Tris" base. EDTA was added to 1 mM. The concentrations, determined from the extinction coefficients, were adjusted to 20 mM and the solutions stored frozen. Working solutions of labelled r- and dNTPs were prepared by adding the 50% ethanol stock labelled material to the corresponding 20 mM unlabelled r- or dNTP and removing the ethanol by evaporation. The volume was adjusted to give a 20 mM solution. Specific activities were

determined by counting samples in Aquasol in the open channel (see below). Specific activities were usually in the range 1200-3000 cpm/nmole.

Resins were prepared according to the manufacturer's directions.

B. Measurement of Radioactivity

All radioactivity was determined in a Beckman LS-250 liquid scintillation spectrometer. Acid-insoluble material was counted by pipetting it on filter paper discs, washing with 5% TCA and ethanol, drying and counting in a toluene-based scintillation fluid (14.4g Omnifluor, New England Nuclear, in 3.8 litres of toluene). Aqueous samples were counted in Aquasol.

Single isotope restricted channels were used for double-labelling experiments and the counts were corrected for overlap. "Open channel" counting refers to counts from an unrestricted channel and includes counts from all isotopes present.

Ratios of [^{3}H]- and [^{14}C]dNMPs in a polymer were determined by counting samples of the polymer in Aquasol using the restricted channels and correcting for overlap. The amount of each dNMP was determined from the specific activity of the original [^{3}H]- or [^{14}C]dNTP which was measured in Aquasol in the appropriate restricted channel.

C. Measurement of Fluorescence

A Turner spectroflurometer Model 430 was used to determine the fluorescence of ethidium bromide bound to DNA. Fluorescence emission was measured at 600 nm using an excitation wavelength of 525 nm. The temperature was regulated at 25° and the fluorometer was frequently calibrated with a standard sample of DNA and ethidium bromide.

D. Purification of E. coli DNA Polymerase I

E. coli DNA polymerase I was purified to Fraction 7 by the method of Jovin et al. , (1969). It was then rechromatographed on phosphocellulose as for Fraction 6 to yield Fraction 8. Essentially all the protein in Fraction 8 migrated as DNA polymerase in SDS gel electrophoresis, i.e. approximately 90% as a protein of 109,000 molecular weight and 10% as a 76,000 molecular weight protein. The latter is a product of proteolytic cleavage, still possessing polymerase and the 3' to 5' exonuclease activities, but lacking the 5' to 3' exonuclease (Brutlag et al. , 1969). The specific activity of Fraction 8 was 10,000 units/ml, one unit being defined as the incorporation of 10 nmoles of total acid-insoluble nucleotide per 30 minutes at 37° with poly[d(A-T)•d(A-T)] as template. The DNA polymerase referred to in all synthetic reactions reported here is Fraction 8.

Fraction IV and Fraction 4 refer to DNA polymerase or S

factor in the same preparation. Fraction IV is the designation used in an earlier preparation of DNA polymerase (Richardson et al. , 1964b). Fraction 4 has a higher protein concentration than Fraction IV but is otherwise identical.

E. Purification of RNA Polymerase

RNA polymerase was prepared to Fraction 3 by the method of Burgess (1969) and further purified as for Fractions III-VI by the method of Paetkau and Coy (1972), omitting the 0.5M Agarose step and substituting DEAE-Sephadex for QAE-Sephadex. One unit of enzyme activity corresponds to the incorporation of 1 nmole labelled rNMP per hour at 37°.

F. Preparation of DIII

DIII was a by-product of a modification (Paetkau and Coy, 1972) of the Chamberlin and Berg (1962) RNA polymerase preparation. DIII designates the material extracted from the protamine pellet with 10 mM Tris-Cl (pH 8) - 0.1 M magnesium chloride - 0.1 mM EDTA - 10 mM 2-mercaptoethanol, precipitated between 45 and 65% ammonium sulfate, and redissolved in 20 mM potassium phosphate (pH 7.4). The protein concentration was 1.8 mg/ml.

DIII contained a partly heat stable form of S factor activity (Coulter et al. , 1974). It was used to prevent clc DNA accumulation (Chapter III). DIII was usually added to synthesis mixtures at 1/200 (v/v) dilution. Heating DIII to

97° for 10 minutes removed most of the nuclease activity. Heated DIII was usually used at 1/20 dilution.

G. Purification of Exonuclease III

Exonuclease III was obtained from a DNA polymerase preparation (Jovin et al., 1969) and was further purified by rechromatography on phosphocellulose and G-100 Sephadex as for Fraction 6 and 7 in the DNA polymerase preparation. The assay was described by Richardson et al. (1964a). A [^{3}H-T]poly[d(A-T)•d(A-T)] template was substituted. A unit of activity is defined as one nmole of dNMP made acid-soluble per 30 minutes at 37°.

The molecular weight of the enzyme was determined by comparison to protein markers of known molecular weight on a calibrated G-100 Sephadex column and on SDS polyacrylamide gel electrophoresis. The values obtained were 35,000±5000 and 15,000±5000 respectively. A second minor component seen on the SDS gels appeared to have a molecular weight less than 10,000. It was not certain whether this low molecular weight material was part of the exonuclease III or was an artefact.

H. Nuclease Assays

(i) Nonspecific nuclease activity

Nonspecific nuclease activity was measured in a reaction mixture consisting of 67 mM Tris-Cl (pH 8) - 1.3 mM

magnesium chloride - 1.2 mM 2-mercaptoethanol - 0.12 A260 [^{3}H-T]poly[d(A-T)•d(A-T)] (155,000 cpm/A260 unit). Aliquots were removed at intervals and placed on filter paper discs for counting. A unit of nonspecific nuclease activity is defined as causing the release of one nmole of nucleotide per minute at 37°.

(ii) Transfer RNA-inhibitable nuclease activity

Nuclease activity inhibitable by tRNA was determined by the method of Flintoff (1973). The substrate was [^{3}H-T,^{14}C-C]poly[d(T-G)•d(C-A)] in the presence and absence of 0.5 A260 tRNA. One nuclease unit was defined as causing one nmole of nucleotide released per minute at 37°.

I. In vitro Copying of DNAs

(i) PM2 RFII

PM2 DNA was copied at 37° in a synthesis mixture consisting of 67 mM potassium phosphate (pH 7.4) - 12 mM magnesium chloride - 2 mM DTT - 0.55 mM each dNTP - 0.5 A260 DNA - 140 units/ml DNA polymerase. S factor and labelled nucleotides were added as indicated.

(ii) Poly[d(T-G)•d(C-A)] and poly[d(T-T-G)•d(C-A-A)]

The procedures for in vitro copying of defined DNAs have been described (Morgan et al., 1974). The synthesis reaction mixture consisted of 30 mM potassium phosphate (pH

7.4) - 12 mM DTT - 12 mM magnesium chloride - 5.2 mM total of the four dNTPs in the molar ratios at which they occur in the given template - 0.2 A260 DNA template - 70 units/ml DNA polymerase. S factor and labelled dNTPs were also present where indicated. Pancreatic DNase I added to stimulate synthesis was usually at 25-50 ng/ml unless otherwise indicated. Transfer RNA was added at 0.5 A260 to inhibit endonuclease I (Lehman et al. , 1962). Transfer RNA was always present in poly[d(T-G)•d(C-A)] synthesis during S factor assays but otherwise only where indicated. The reaction mixture was incubated at 37°. Under these conditions at least 30-fold copying occurred in 5 hours.

Synthesis of poly[d(T-G)•d(C-A)] was also performed using the decadeoxynucleotides, oligo[d(T-G)] and oligo[d(C-A)]. The synthesis mixture was identical to that above except that 0.2 A260 of each oligodeoxynucleotide supplied the template, heated DIII was added at 1/20 (v/v), tRNA was present, and DNase I was omitted. This resulted in about 50-fold synthesis in 4 hours.

(iii) Poly[d(A-T)•d(A-T)]

The synthesis mixture for poly[d(A-T)•d(A-T)] consisted of 67 mM potassium phosphate (pH 7.4) - 1.4 mM 2-mercaptoethanol - 0.2 mM TTP - 0.2 mM dATP - 6.7 mM magnesium chloride - 7 units/ml DNA polymerase. Template was provided by authentic poly[d(A-T)•d(A-T)] at indicated

concentrations or by various S factor fractions containing the putative poly[d(A-T)•d(A-T)] template. Transfer RNA was present where indicated.

(iv) Determining the extent of synthesis

The extent of synthesis for both natural and chemically defined DNAs was determined either by the conversion of labelled dNTP into TCA-insoluble form measured on filter paper or by comparison to a standard DNA in the ethidium bromide fluorescence assay (described in section K below).

(v) Isolation of defined polymers

Polymers were isolated where indicated by chromatography on 15M Agarose (0.9x35 cm) (Morgan et al., 1974) after the addition of EDTA to twice the magnesium concentration and Sarkosyl or SDS to 0.1%. The elution buffer consisted of 5 mM Tris-Cl (pH 8) - 0.1 mM EDTA. NaCl at 20 mM was present where tRNA had been used in the copying since it resulted in separation of the tRNA from the excluded material. The excluded material was concentrated by vacuum dialysis in collodion bags versus 5 mM Tris-Cl (pH8) - 0.1 mM EDTA and was stored frozen in this buffer.

(vi) Preparation of single strands of poly[d(T-G)] and poly[d(C-A)]

The strands of poly[d(T-G)•d(C-A)] were separated in a preparative alkaline CsCl density gradient. The polymer was synthesized in the standard way with 1/20 heated DIII, isolated, added to an alkaline CsCl solution and centrifuged as shown in Figure 6. Fractions 3 to 8 and 16 to 21 inclusive were pooled separately to obtain poly[d(T-G)] and poly[d(C-A)] respectively. The pooled fractions were neutralized, dialyzed, and concentrated by vacuum dialysis versus 5 mM Tris-Cl (pH 8) - 0.1 mM EDTA. In this case 1.37 A260 units of poly[d(T-G)] and 1.08 A260 units of poly[d(C-A)] were recovered. Radioactively labelled strands were isolated in the same way except that the positions of the strands were determined by the presence of TCA-insoluble radioactivity.

J. Transcription by RNA polymerase

The reaction mixture for RNA polymerase reactions consisted of 40 mM Tris-Cl (pH 8) - 4 mM magnesium chloride - 0.8 mM manganese chloride - 10 mM 2-mercaptoethanol - 50 mM KCl - 0.2 A260 DNA template - 600 units/ml RNA polymerase. Ribonucleoside triphosphates were present at 0.30 mM each for templates having equimolar fractions of each base; 0.25 mM and 0.50 mM for templates having 1:2 molar fractions of the corresponding bases. The extent of

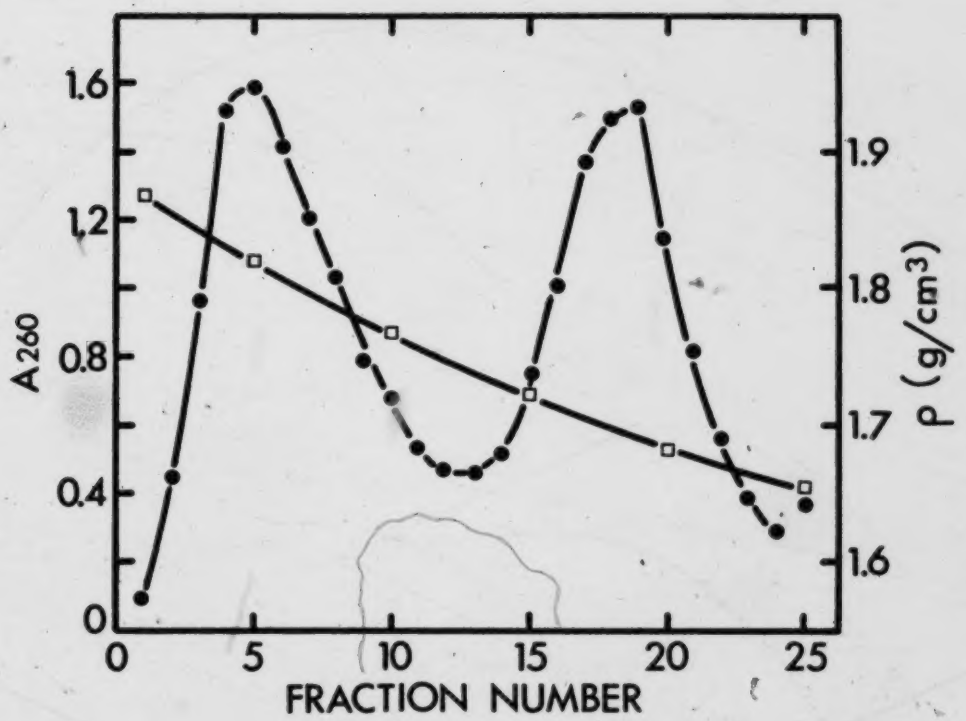


FIGURE 6. Separation of Strands of Poly[d(T-G)•d(C-A)] by Preparative Alkaline CsCl Density Gradient Centrifugation. The polymer (3.5 A260 units) was added to a solution of 50 mM NaOH - 1 mM EDTA with CsCl to give a density of 1.778 g/cm³. The solution was centrifuged in an SW 65L Ti rotor at 38,000 rpm for 69 hours at 20°. Fractions (0.2 ml) were pumped from the bottom of the tube. Densities were determined refractometrically. The absorbance of each fraction was determined in 1/10 dilutions. Circles, A260; squares, density.

transcription was monitored by incorporation of ^{14}C-labelled rNTPs (specific activities in the range 2000-2500 cpm/nmole) into TCA-insoluble material. This general procedure for transcription was used to obtain different sorts of information in the following ways:

a). Molar ratio of bases in the template. The template was transcribed in the presence of the 4 rNTPs. The relative incorporation of the 2 rNTPs complementary to one strand of the template was determined by comparing parallel reaction mixtures containing one or both of these rNTPs labelled, and otherwise identical.

b). Base content of a template strand. The incorporation of a single labelled rNTP in the presence of each of the other three in turn was compared with the incorporation of the labelled rNTP by itself. Dependence of incorporation of a given nucleotide on the presence of another nucleotide was taken as evidence that the pair occurred within the same strand of the template.

c). Homopolymer synthesis. Labelled rGTP or rCTP was the only nucleotide present. Transcription to poly[r(G)] is indicative of a polypyrimidine•polypurine template; poly[r(C)] synthesis of extensive poly[d(G)] DNA (Paetkau et al., 1972).

K. Ethidium Bromide Fluorescence Assays

The enhanced fluorescence of ethidium bromide when it is bound to double-stranded nucleic acids (LePecq and Paoletti, 1967) was used in an assay specific for double-stranded DNA (Morgan and Paetkau, 1967). The buffers used in this assy were: TE buffer, 2 mM Tris-Cl (pH 8) - 0.2 mM EDTA for defined DNAs; or KE buffer, 20 mM tripotassium phosphate (pH 12) - 0.2 mM EDTA for natural DNAs. The KE buffer eliminated the nonspecific structures found in denatured, non-clc natural DNAs (Morgan and Paetkau, 1972). Ethidium bromide was added to a final concentration of 0.5 ug/ml (yielding TEE and KEE buffers respectively) for fluorescence readings. Samples of synthesis mixtures were added directly to the KE or TE buffers since there is no interference from the components of the synthesis mixture. The sample size was adjusted to give a DNA concentration of 0.01 to 0.04 A260 in the assay. There were two main uses made of the fluorescence assay:

a). Monitoring DNA synthesis. Samples removed from synthesis mixtures were added to 3 ml of TE (or KE) buffer. Ethidium bromide was added and the fluorescence measured. The DNA concentration of the sample was determined by comparison with a standard sample of DNA. This method was also used for specifically measuring the DNA content of various enzyme fractions.

b). Measuring clc DNA content. Duplicate DNA samples

were added to 3 ml of TE (or KE) buffer. One sample was heated in boiling water for 5 minutes then quickly cooled in ice. Ethidium bromide was added to both samples and the fluoresence of each measured. Comparison with a standard DNA sample gave the double-stranded DNA content of each sample. The ratio of fluorescence in the heated sample to that in the unheated sample gave the fraction of clc DNA. At the low ionic strength used in the TE and KE buffer, DNA strands separated by heat denaturation are kept apart by ionic repulsion unless there is a covalent bond between complementary strands to act as a nucleation site for reannealing. This is illustrated for various clc structures in Figure 5 (Chapter I).

L. Assay for S Factor Activity

The assay for S factor activity was a direct application of clc DNA measurements by the fluorescence assay. Poly[d(T-G)•d(C-A)] was synthesized in the usual way in the presence of 0.5 A260 tRNA and various concentrations of S factor fractions. After 5 hours aliquots were assayed for clc DNA. A unit of S factor activity is defined as causing a 50% reduction in clc poly[d(T-G)•d(C-A)] content (Flintoff and Paetkau, 1974). The relationship between the amount of S factor added and the reduction in the percent clc DNA is not a simple one (Figure 18a, Chapter IV). The curve must often be extrapolated to reach the 50% reduction

level.

M. Protein Determinations

Protein concentrations were measured by absorbance at 280 nm or by a modified Biuret reaction (Lane and Mavrides, 1969).

N. Dilution Buffers

The following dilution buffers were used: for DNA polymerase, S factor, and DNase I, 0.3 mg/ml BSA - 50 mM potassium phosphate (pH 7.4) - 0.5 mM DTT; for endonuclease I, 0.25 M ammonium sulfate - 50 mM Tris-Cl (pH 7.5) - 1 mg/ml BSA.

O. Molar Extinction Coefficients of Defined DNAs

The following extinction coefficients (nucleotide equivalents) were used for determining DNA concentrations: poly[d(A-T)•d(A-T)], 6.7×10^3 (Inman and Baldwin, 1962); poly[d(T-G)•d(C-A)], 6.5×10^3 (Wells *et al.*, 1970); and 9×10^3 for single strands of poly[d(T-G)] and poly[d(C-A)] (35% hyperchromicity on denaturation).

P. Batchwise Hydroxyapatite Analysis

Hydroxyapatite can be used to differentiate single- and double-stranded DNA and therefore DNA which is renaturable (Bernardi, 1971). A batchwise method was used to detect clc DNA in natural DNAs copied *in vitro*. A sample of labelled

DNA was added to a 1.5 ml mixture containing 85 mM potassium phosphate (pH 6.8) - 0.2 A260 calf thymus carrier DNA. The mixture was heated in boiling water for 5 minutes and quickly cooled on ice. An equal amount of native calf thymus DNA carrier was added [redacted] A260). One ml of 40% hydroxyapatite suspension was added and mixed. After 5 minutes the supernatant was diluted to 5 ml with 50 mM potassium phosphate (pH 6.8) and removed. The hydroxyapatite was extracted three times with 50 mM potassium phosphate, then with 0.10 M potassium phosphate until aliquots counted in Aquasol indicated that all unadsorbed material had been removed. Single-stranded DNA was eluted by two extractions with 0.16 M potassium phosphate; double-stranded DNA by two extractions with 0.24 M potassium phosphate. The percentage of total DNA in each fraction was determined by counting aliquots in Aquasol.

Q. Formic Acid-Diphenylamine Degradation

Formic acid-diphenylamine hydrolysis was performed by the method of Burton (1967). Poly[d(A-T)•d(A-T)] at 1.0-1.7 A260 was incubated 13 hours at 30° with 2 volumes of 98% formic acid - 3% diphenylamine. Diphenylamine was removed by 3 extractions with 1 volume of water and 12 volumes of ether. The free purine bases were removed by chromatography on Dowex 50WX-4 columns (0.5x8 cm). The free pyrimidine nucleotides were eluted with water and repeatedly concentrated from water to yield a sample at neutral pH. For

bacterial alkaline phosphatase treatment the sample was made 50 mM in Tris-Cl (pH 8) and 5 mM in magnesium chloride. Alkaline phosphatase was added to 38.5 ug/ml and the mixture was incubated 6 hours at 37°, then subjected directly to electrophoresis.

Electrophoresis was performed using Whatman 3 MM paper prewashed with 0.1 M sodium citrate (pH 4.15) - 1 mM EDTA and dried before use. The electropherogram was run with the same buffer on a 45 cm plate at 1500V for 1.5 hours at 22°. The positions of the resulting spots were determined by absorption of UV light. The labelled components were detected and measured by cutting the paper into 1.5x2 cm strips which were soaked in 1 ml of water in scintillation vials at 65° for 30 minutes. Ten ml of Aquasol was added to each for counting. This procedure eluted the labelled components from the paper and overcame the loss of counting efficiency caused by the citrate in the usual toluene-based scintillation fluid.

R. Temperature-Absorbance Profiles

Tm's were measured in a Gilford Spectrophotometer (2400) with a Haake circulating heater. Solutions were placed in stoppered one ml cuvettes. The composition of the buffers varied and is indicated where they were used. DNA was added to an intial concentration of approximately 0.2 A260. The rate of temperature increase was about 0.5-1.0

degree/minute. The temperature and absorbance were displayed on a chart recorder. The Tm was taken as the midpoint of the increase in absorbance.

S. Ultracentifugation

(i) Preparative scale equilibrium centrifugation

Centrifugation was performed in a Beckman L2-65B Ultracentrifuge, using new polyallomer tubes. The gradients were fractionated by lowering a needle to the bottom of the tube and pumping fractions with an LKB peristaltic pump at a rate of 0.4 ml/minute. Densities were determined from refractive index by comparison to a standard curve.

(ii) Analytical scale ultracentrifugation

Analytical ultracentrifugation was performed in a Beckman Model E ultracentrifuge with UV optics. Tracings of photographs were obtained with a Joyce-Loebel microdensitometer.

For equilibrium banding a cell having a 4°, 12 mm Kel-F centerpiece and a -1° wedge window was used. The solutions consisted of 0.2 A260 DNA - 1 mM EDTA - CsCl to the desired density, with 20 mM NaOH added for alkaline gradients. The solutions were centrifuged at 48,000 rpm for approximately 20 hours at 20° unless otherwise indicated. The density of the DNA at equilibrium was calculated by the isoconcentration method (Vinograd, 1963) using the beta

values determined by Ifft et al. (1961).

Molecular weights of DNAs were determined by velocity sedimentation according to the method of Studier (1965). The cell used was a Vinograd type 1 with a 4°, 12 mm Kel-F centerpiece with quartz windows. The DNA sample (20 ul at 2 A260) was layered onto the solvent at low speed, then centrifuged at 56,000 rpm and 25°. Photographs were taken at 8 minute intervals. The solvent consisted of 1 M NaCl - 0.035 M disodium phosphate - 0.015 M monosodium phosphate - 0.1 mM EDTA, pH 6.7 for neutral runs; and 0.9 M NaCl - 0.1 M NaOH - 0.1 mM EDTA for alkaline runs. The DNA sample was in 0.2 M NaOH for alkaline sedimentation. The sedimentation coefficient was calculated from the tracings of the photographs and was converted to molecular weight using the equations of Studier (1965). The equation for alkaline sedimentation has recently been shown to be valid for polynucleotides of the lengths encountered in this work (Hirose et al., 1973).

T. Polyacrylamide Gel Electrophoresis

Polyacrylamide gels (0.5x6.5 cm) at 5% and 10% acrylamide were prepared according to the specifications given in Canalco Chemical Formulations for Disc Electrophoresis (1968). The gels were buffered with 50 mM sodium phosphate (pH7.2) and contained 0.1% SDS.

The protein solutions were used directly if their salt

content did not raise the final concentration in the sample above 50 mM. Otherwise they were dialyzed versus 5 mM sodium phosphate (pH 7.2) and concentrated by evaporation. The samples consisted of 5-10 ug protein -10 mM DTT - 0.33% SDS. They were heated at 85° for 15 minutes to denature the proteins. Glycerol was added to 20% and the samples applied to the gels by layering under the electrophoresis buffer (0.1% SDS - 50 mM sodium phosphate, pH 7.2). The gels were run at 5 ma/gel for 1.5 to 2 hours . The gels were removed from the tubes and stained for 2 hours at 37° in 0.24% Coomassie blue - 9.8% acetic acid - 45% methanol (Burgess, 1969). They were destained in 7% acetic acid in a Canalco horizontal destainer. The gels were then placed in fresh 7% acetic acid and incubated in the dark at 37° for 24 to 48 hours before scanning. This reduced the background essentially to zero. The gels were scanned at 540 nm in a Gilford spectrophotometer with a linear transport attachment.

A plot of the log of the molecular weights of standard proteins versus their fractional migration distances yielded a standard curve from which molecular weights of unknowns can be determined (Shapiro et al. , 1967). For molecular weight determinations of S factor (Chapter IV) the standards used were ovalbumen (45,000), chymotrypsinogen A (26,000), R17 A protein (39,000), and R17 coat protein (13,750). The R17 proteins were derived from whole bacteriophage lysed

during the protein denaturation step.

Tracings of gels were also used to determine the percentage composition of various components by comparison of the areas of the bands.

CHAPTER III

SYNTHESIS OF DEFINED DNAS AND CLC DNA PRODUCTION

I Introduction

During the *in vitro* copying of DNA polymers of defined sequence by *E. coli* DNA polymerase I, structures arise which have covalent links between complementary sequences. Such structures are referred to as clc DNA (Morgan and Paetkau, 1972). The existence of such clc DNA was first indicated by the "non-denaturability" of DNA copied *in vitro* by DNA polymerase (Schildkraut *et al.*, 1964). The DNA polymer, poly[d(T-G)•d(C-A)] was also seen to produce clc structures when copied by DNA polymerase (Paetkau, 1969).

The detection of clc sequences in the defined DNA polymers, poly[d(A-T)•d(A-T)], poly[d(T-G)•d(C-A)], and poly[d(T-T-G)•d(C-A-A)] by the ethidium bromide fluorescence assay and/or alkaline CsCl density gradient centrifugation is described in this chapter. A special problem was presented by poly[d(T-T-G)•d(C-A-A)] in that the available template already contained clc sequences. In order to demonstrate the production of clc DNA from non-clc templates it was necessary to generate a non-clc template. A detailed study of the *in vitro* synthesis and detection of clc DNA has

been reported (Coulter et al. , 1974).

II Results

Unless otherwise indicated all polymers were copied and isolated according to the methods described in Chapter II.

A. Production of clc DNA in Chemically Defined DNA Polymers

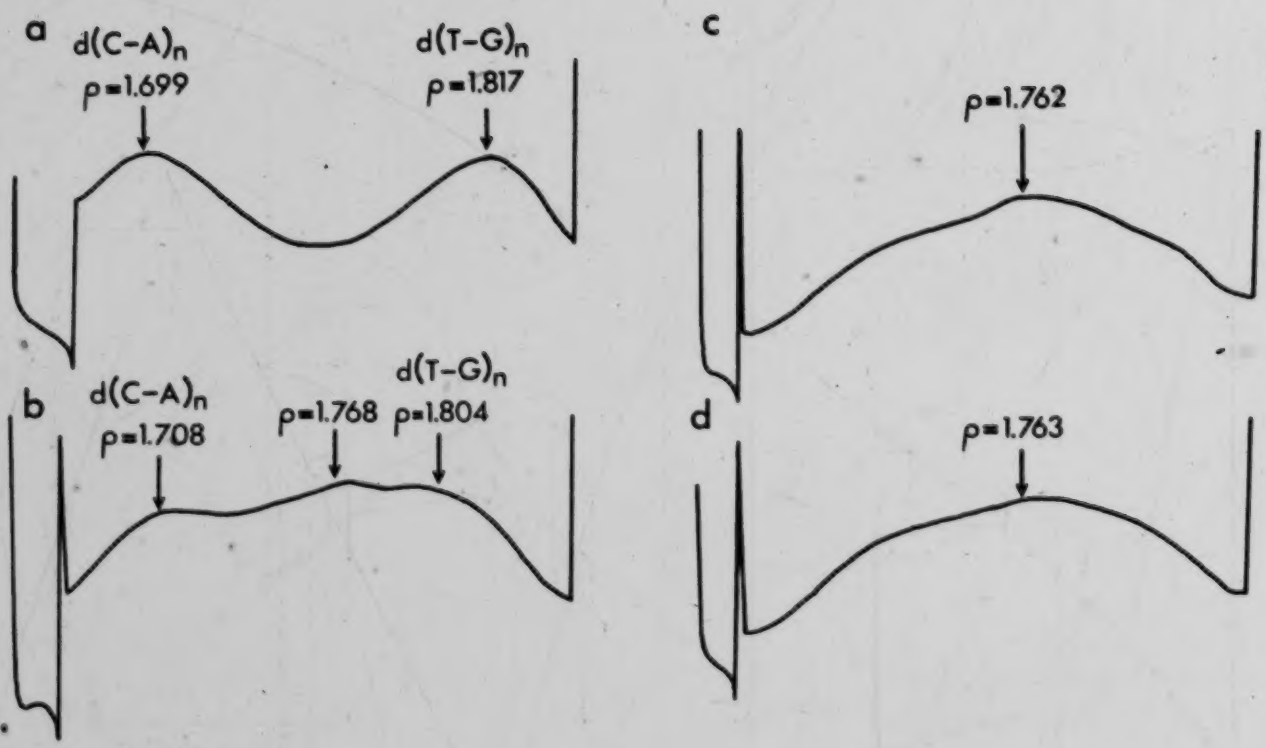
(i) Poly[d(A-T)•d(A-T)]

The polymer poly[d(A-T)•d(A-T)] consists of 100% clc structures by definition (Chapter V, Table XI). It would be predicted to undergo completely reversible denaturation and this was always found to be the case (Morgan and Paetkau, 1972).

(ii) Poly[d(T-G)•d(C-A)]

Separable-stranded poly[d(T-G)•d(C-A)] was copied in the usual synthetic mixture with and without the addition of 9 ug/ml DIII (Chapter II). After 5 hours the reaction was stopped and the products isolated by Agarose chromatography. Analytical equilibrium centrifugation in alkaline CsCl was used to determine the presence or absence of clc DNA as shown in Figure 7a,b. The polymer prepared in the absence of DIII showed material at a density intermediate between that of the poly[d(T-G)] and poly[dC-A)] strands (Figure 7b). This is the clc product. The product prepared in the

FIGURE 7. Analytical Alkaline CsCl Density Gradient Centrifugation of Poly[d(T-G)•d(C-A)]. Polymers were copied from non-clc templates (a,b), or clc templates (c,d), with (a,d) or without (b,c) addition of DIII. After isolation the polymers were centrifuged in alkaline CsCl as described in Chapter II. Densities were calculated by the isoconcentration method.


a
d(C-A)n
ρ=1.699
d(T-G)n
ρ=1.817
b
d(C-A)n
ρ=1.708
ρ=1.768
d(T-G)n
ρ=1.804
c
ρ=1.762
d
ρ=1.763

presence of DIII contained essentially no clc product. The results of a similar experiment using a template already containing clc structures are shown in Figure 7c,d. In this case both products contained clc structures. The molecular weights of the four polymers prepared above are given in Table IIIa. The presence of nuclease activity in DIII was indicated by the lower molecular weight of the products made with DIII present.

In separate experiments the clc DNA content of the poly[d(T-G)•d(C-A)] products was measured by the fluorescence assay. The results, which are in agreement with alkaline CsCl equilibrium analyses, are shown in Table IIIb. It was also shown that the copying of the decadeoxynucleotides oligo[d(T-G)] and oligo[dC-A)] in the absence of DIII resulted in clc DNA production. The polymers made in the absence of DIII often show a percentage of clc above zero when measured by the fluorescence assay. This background varies from one polymer preparation to another and may represent low molecular weight clc DNA which is not copied during subsequent synthesis in the presence of DIII or S factor.

It has been shown (Coulter et al., 1974) that during the copying of non-clc poly[d(T-G)•d(C-A)] by DNA polymerase in the absence of S factor clc DNA increases in parallel with the increase in total DNA. Clc DNA was detectable after about twofold copying of the template had occurred.

TABLE III

Characteristics of Polymers Copied from clc and Non-clc Templates

a) Molecular Weights

Nature of Template	DIII	Molecular Weights[1] SS	DS	Ratio[2] SS/DS
non-clc	+	62,000	204,000	0.30
	-	116,000	225,000	0.52
clc	+	66,000	162,000	0.42
	-	100,000	191,000	0.52

[1] Molecular weights determined by sedimentation velocity ultracentrifugation (Chapter II).
[2] Determined from molecular weights.

b) Clc Content Measured by Ethidium Bromide Fluorescence

Template	DIII	%clc
non-clc poly[d(T-G)•d(C-A)] [1]	+	0
	-	19.5
oligo[d(T-G)] + oligo[d(C-A)] [2]	+	5.5
	-	34.5

[1] DIII at 9 ug/ml. Clc measured at 4 hours.
[2] DIII at 18 ug/ml. Clc measured at 6 hours.

(iii) poly[d(T-T-G)•d(C-A-A)]

The polymer, poly[d(T-T-G)•d(C-A-A)] was copied and isolated by the usual methods. The initial template and the product both contained 30% clc sequences as measured by the fluorescence assay. Analytical equilibrium centrifugation of the product in alkaline CsCl showed a broad band centered at 1.757 g/cm^3 as shown in Figure 8. As with poly[d(T-G)•d(C-A)], the ethidium bromide fluorescence assay and alkaline CsCl density gradient centrifugation results agreed qualitatively.

B. Production of Non-clc Poly[d(T-T-G)•d(C-A-A)]

(i) Separation of strands of poly[d(T-T-G)•d(C-A-A)]

The segregation of ionizable bases in poly[d(T-T-G)•d(C-A-A)] and experience with other defined polymers indicated that the poly[d(T-T-G)] and poly[d(C-A-A)] strands should be separable in an alkaline CsCl density gradient. In order to amplify the difference in density between the two strands BrdUTP was incorporated as a heavy label. A sample of poly[d(T-T-G)•d(C-A-A)] containing 30% clc DNA was copied in the standard conditions using BrdUTP (1.8 mM) with [^{14}C]dCTP and a trace of [^{3}H]TTP (0.2 uM) as labels. The specific activities of the labels were 1550 cpm/nmole and 1350 ^{3}H cpm/nmole BrdUTP respectively. The replacement of TTP by BrdUTP resulted in 40-50% inhibition of synthesis. The reaction mixture and the synthetic products were

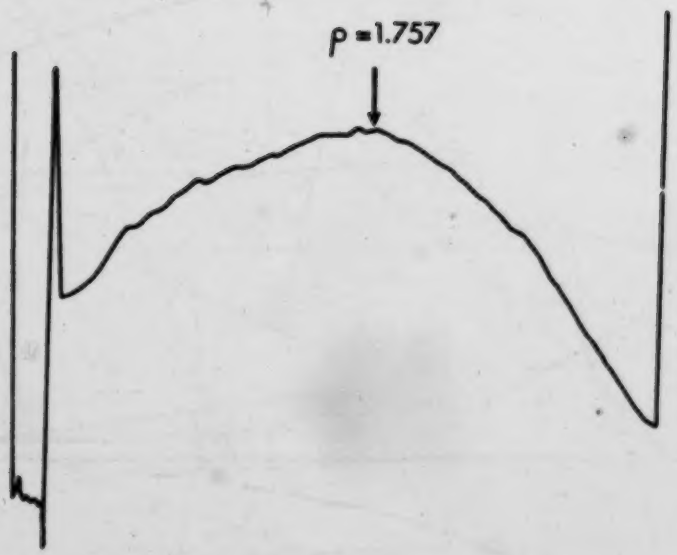


FIGURE 8. Analytical Alkaline CsCl Density Gradient Centrifugation of Poly[d(T-T-G)•d(C-A-A)] Containing clc Sequences. The polymer was centrifuged as described in Chapter II. Density was determined by the isoconcentration method.

shielded from the light. Synthesis was monitored by the ethidium bromide fluorescence assay and was stopped after 7 hours by addition of EDTA to 30 mM and Sarkosyl to 0.1%. The product was isolated and then subjected to preparative cesium sulfate equilibrium centrifugation as shown in Figure 9.

Fractions 4 to 7 and 22 to 24 inclusive were pooled separately, and neutralized, dialyzed and concentrated by vacuum dialysis versus 5 mM Tris-Cl (pH 8)-0.1 mM EDTA. The fractions pooled were assumed to consist of [^{3}H-T]poly[d(BrU-BrU-G)] and [^{14}C-C]poly[d(C-A-A)] respectively on the basis of their labelling. The ^{3}H-T label occurs with low frequency in the poly[d(BrU-BrU-G)] chain. There was 10-20% cross-contamination of label between the separate strands. The material banding at an intermediate density of 1.52 g/cm^{3} contained both radioactive labels and was assumed to consist of [^{3}H-T]poly[d(BrU-BrU-G)] covalently linked to [^{14}C-C]poly[d(C-A-A)] i.e. clc DNA.

(ii) Synthesis of non-clc poly[d(T-T-G)•d(C-A-A)]

The separated strands of poly[d(BrU-BrU-G)•d(C-A-A)] were used to produce a double-stranded non-clc polymer. The strands, at 0.2 A260 in 5 mM Tris-Cl (pH 8)-0.1 mM EDTA, were annealed by heating them in boiling water for 2 minutes then slowly cooling (45 minutes) to room temperature. The annealed product was added to the usual DNA polymerase

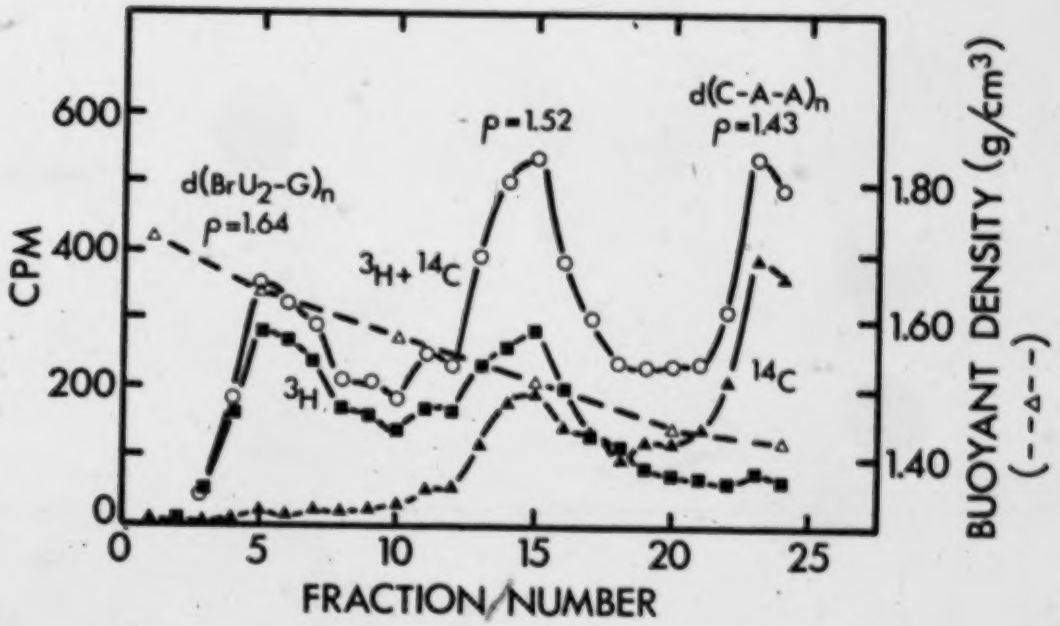


FIGURE 9. Preparative Alkaline Cesium Sulfate Density Gradient Centrifugation of Poly[d(BrU-BrU-G)•d(C-A-A)]. Centrifugation was performed in the a Ti 50 rotor at 40,000 rpm for 65 hours at 20°. Fractions (0.2 ml) were pumped from the bottom. Aliquots were removed for determining TCA-insoluble cpm in restricted and open channels. Densities were determined refractometrically.

reaction mixture containing DIII. The product was labelled with [^{14}C]dCTP and [^{3}H]dATP at specific activities of 1350 and 1180 cpm/nmole respectively. The extent of synthesis was monitored by the fluorescence assay, with the results shown in Figure 10. The reaction was stopped at 7 hours by the addition of EDTA and Sarkosyl and the product was isolated. Analytical equilibrium centrifugation in alkaline CsCl showed predominantly separable-stranded material (Figure 11).

(iii) Characterization of non-clc poly[d(T-T-G)•d(C-A-A)]

It was necessary to demonstrate that the preparation contained only poly[d(T-T-G)•d(C-A-A)] because of the problems to be discussed below (section D). Three methods were used in this characterization:

a). Temperature-absorbance profile. The Tm was obtained as described in Chapter II. It was 69° in 1/10 SSC, with 35% hyperchromicity in agreement with the literature value (Wells et al., 1970).

b). Ratio of [^{14}C]dCTP/[^{3}H]dATP. The ratio was determined by counting a sample of the polymer in Aquasol using the restricted single isotope channels as described in Chapter II. A sample of similarly labelled poly[d(T-G)•d(C-A)] was used for comparison. The ratio of ^{14}C-dC to ^{3}H-dA for poly[d(T-T-G)•d(C-A-A)] was half that for poly[d(T-G)•d(C-A)], as expected.

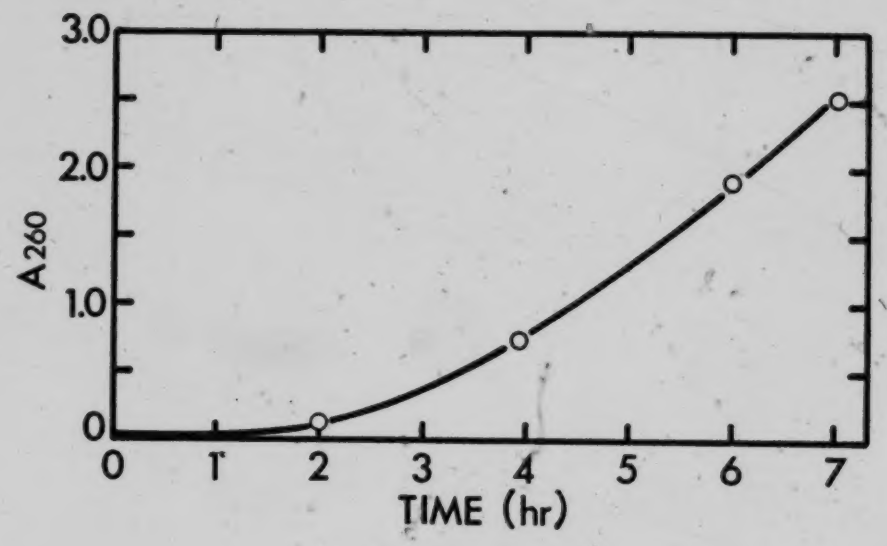


FIGURE 10. Synthesis of Poly[d(T-T-G)•d(C-A-A)] from Annealed Strands of Poly[d(BrU-BrU-G)] and Poly[d(C-A-A)]. The annealed strands were diluted 1/7 into a synthesis mixture with 0.5 ng/ml pancreatic DNase I, 0.5 A260 tRNA, and 1/20 v/v heated DIII. Synthesis was monitored by the fluorescence assay, and converted to A260 units by comparison to a standard DNA.

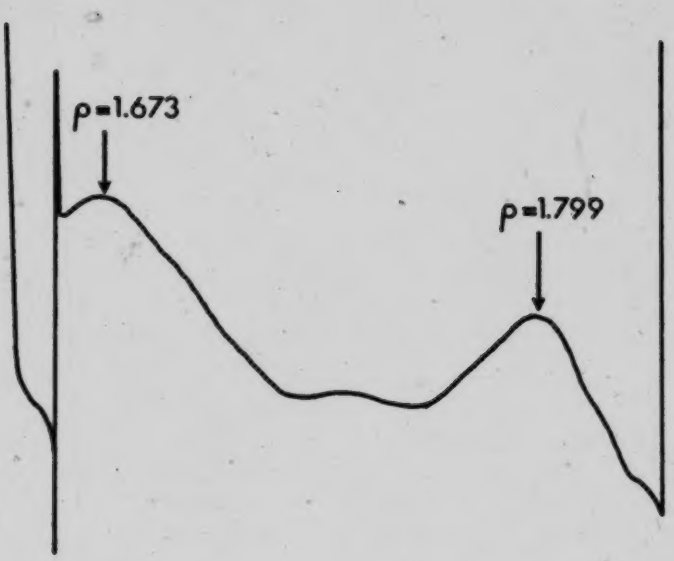


FIGURE 11. Analytical CsCl Density Gradient Centrifugation of Poly[d(T-T-G)•d(C-A-A)] Synthesized from Annealed Strands of Poly[d(BrU-BrU-G)] and Poly[d(C-A-A)]. The polymer was prepared in the presence of DIII as described in Figure 10 and centrifuged in the usual way (Chapter II). Densities were determined by the isoconcentration method. The initial density was 1.750 g/cm^3.

c). Transcription with RNA polymerase. Transcription of poly[d(T-T-G)•d(C-A-A)] (Chapter II) was performed using various combinations of rNTPs. The results are shown in Table IV. There was no poly[r(G)] synthesis when [^{14}C]rGTP was the only nucleotide present. The incorporation of [^{14}C]rCTP was dependent on the addition of rATP but not rGTP or rUTP. Similarly the incorporation of [^{14}C]rUTP was stimulated by the addition of rGTP but not rATP or rCTP (a variable background and a low level of total label incorporated account for the apparent incorporation in the absence of rGTP). The ratio of [^{14}C]rCTP/[^{14}C]rATP incorporated was 1/2.

All of these procedures confirmed that the polymer preparation was poly[d(T-T-G)•d(C-A-A)]. The template was copied at a rate sufficient to avoid copying template material in DIII.

(iv) Effect of endonuclease I treatment on clc structures in poly[d(T-T-G)•d(C-A-A)]

Partial endonuclease I degradation was used in an attempt to decrease the amount of clc DNA in a poly[d(BrU-BrU-G)•d(C-A-A)] polymer. It was expected that double-stranded cuts made by this enzyme might release material from the covalent linkage between complementary sequences. The polymer was prepared and isolated as before. The

TABLE IV

Transcription of Poly[d(T-T-G)•d(C-A-A)] using Various Combinations of Ribonucleotides

rNTPs Present	Incorporation in 30 Minutes nmoles /ml
[^{14}C]rGTP[1]	0
[^{14}C]rCTP[2]	0
+ rATP	4.2
+ rGTP	0
+ rUTP	0
[^{14}C]rUTP[3]	1.7
+ rATP	2.8
+ rGTP	7.3
+ rCTP	2.2

[1]Specific activity, 1900 cpm/nmole.
[2]Specific activity, 2600 cpm/nmole.
[3]Specific activity, 2000 cpm/nmole.

poly[d(BrU-BrU-G)•d(C-A-A)], at 2 A260, was incubated at 37° in a reaction mixture consisting of 67 mM Tris-Cl (pH 8) - 6.7 mM magnesium chloride - 7 units/ml endonuclease I. Samples removed at various times were made 16 mM in EDTA, heated in boiling water for 2 minutes, and then subjected to preparative alkaline cesium sulfate density gradient centrifugation exactly as before. The distribution of TCA-insoluble cpm was similar to that seen in Figure 9. Incubation with endonuclease I decreased the percentage of material occurring in the center band, i.e. clc DNA from 50% to 30%. This is to be expected if double-stranded cleavages by the enzyme are random. The effect did not appear to be specific to the clc DNA since all three bands were broadened and there was loss of resolution with increasing incubation time. Partial endonuclease I digestion of poly[d(T-T-G)•d(C-A-A)] increased the incidence of anomolous sequences in subsequent copying (section D) and so was not used as a treatment of templates prior to copying with DNA polymerase.

C. Non-clc Poly[d(T-T-G)•d(C-A-A)] as a Template

Non-clc poly[d(T-T-G)•d(C-A-A)] was used as a template for subsequent copying by DNA polymerase in the usual reaction mixture. In one case 100 ng/ml pancreatic DNase I, 20 ug/ml purified S factor (free of poly[d(A-T)•d(A-T)] template activity, Chapter II), and 0.5 A260 tRNA were also present. In a second case only DNase I was added. The synthesis was monitored by fluorescence. The reactions were

stopped and the products isolated, then examined by analytical alkaline CsCl density gradient centrifugation as shown in Figure 12. The polymer made in the presence of S factor showed two well-separated bands at densities of 1.671 g/cm^3 and 1.803 g/cm^3 (Figure 12b). The polymer prepared in the absence of S factor showed the two outer bands and a band of intermediate density at 1.742 g/cm^3 (Figure 12a). The intermediate band represents clc DNA. Fluorescence measurements showed 16% clc content for the polymer made with S factor and 37% for the one made without S factor. The high percent clc for the former in this case may be due to low molecular weight clc material which is detected by the fluorescence assay but not by alkaline CsCl density gradients. Such material does not appear to serve as a template. Both polymers showed Tm's which agreed with the literature value for poly[d(T-T-G)•d(C-A-A)] (Wells et al., 1970).

D. Defined DNA Polymers as Templates for Further Copying

(i) Occurrence and detection of incorrect sequences arising during copying of poly[d(T-G)•d(C-A)]

The copying of poly[d(T-G)•d(C-A)] was usually accurate and presented no problems of fidelity unless high concentrations of crude S factor were present. Such fractions contained sufficient poly[d(A-T)•d(A-T)] template (see Chapters IV and V) that poly[d(A-T)•d(A-T)] was

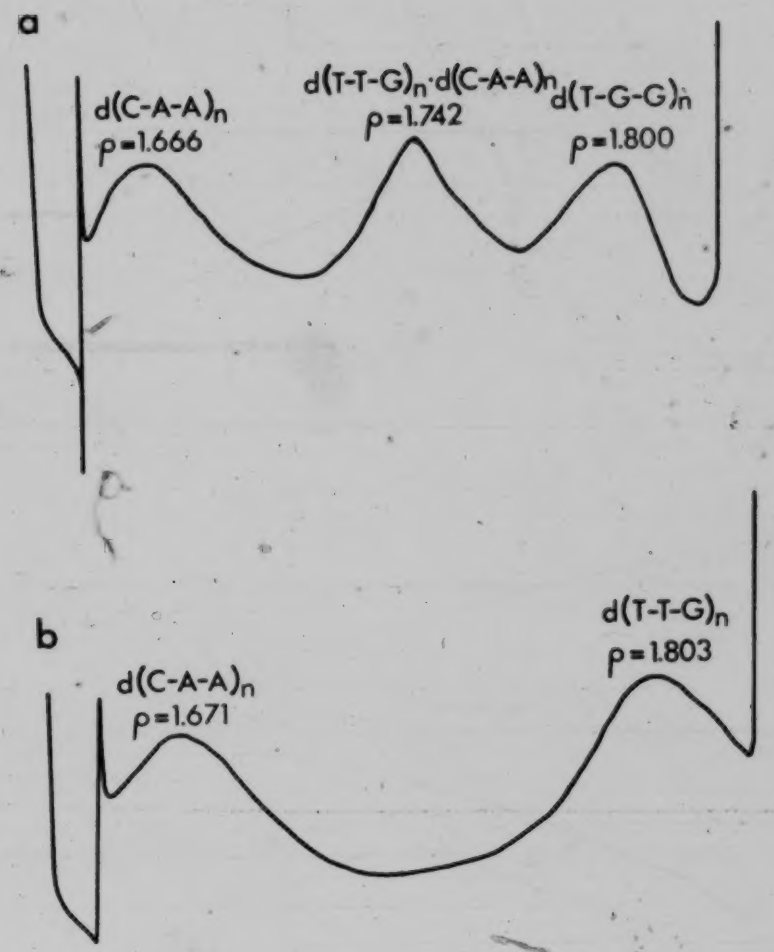


FIGURE 12. Analytical Alkaline CsCl Density Gradient Centrifugation of Poly[d(T-T-G)•d(C-A-A)]. Polymers were prepared either with (b) or without (a) purified S factor. The isolated polymers were centrifuged as usual for 45 hours. Densities were determined by the isoconcentration method. The initial density was 1.742 g/cm³.

produced along with poly[d(T-G)•d(C-A)]. The effect of various concentrations of crude S factor on the product composition during the copying of poly[d(T-G)•d(C-A)] by DNA polymerase will be described more fully in Chapter IV.

The production of poly[d(A-T)•d(A-T)] during poly[d(T-G)•d(C-A)] copying was readily detectable by two methods:

a). Ratio of [^{14}C]dCMP/[^{3}H]TMP incorporated and percent clc DNA in the product. The synthesis of poly[d(A-T)•d(A-T)] along with poly[d(T-G)•d(C-A)] resulted in a decrease in the amount of [^{14}C]dCMP incorporated relative to the amount of [^{3}H]TMP, with a concomitant increase in the percent clc DNA measured by the fluorescence assay.

b). Analytical alkaline CsCl density gradient centrifugation. The formation of a distinct and usually very sharp band at a density of 1.722 g/cm^3 revealed the presence of contaminating poly[d(A-T)•d(A-T)]. The poly[d(T-G)] and poly[d(C-A)] strands were well-resolved from the poly[d(A-T)•d(A-T)] at densities of 1.825 g/cm^3 and 1.684 g/cm^3 respectively (Wells and Blair, 1967).

(ii) Effectiveness of poly[d(T-T-G)•d(C-A-A)] as a template for further synthesis

The copying of poly[d(T-T-G)•d(C-A-A)] by DNA polymerase was compared to the copying of poly[d(T-G)•d(C-

A)]. Two different poly[d(T-T-G)•d(C-A-A)] templates were used: A, which contained 30% clc DNA, and B, which contained 15% clc DNA. B was prepared by copying A after endonuclease I pretreatment. The compositions of both A and B were found to be essentially 100% poly[d(T-T-G)•d(C-A-A)]. The results are shown in Figure 13. The ratios of [^{14}C]dCMP/[^{3}H]dAMP incorporated were 1.69 for poly[d(T-G)•d(C-A)] and 0.82 and 0.83 for A and B respectively. There was essentially no lag period for the poly[d(T-G)•d(C-A)] template and incorporation was linear for 7 hours. On the other hand, there was a lag period of 1 to 2 hours for template A and 4 to 5 hours for template B before the incorporation approached a linear rate. The poly[d(T-G)•d(C-A)] appeared to be utilized as a template more effectively than poly[d(T-T-G)•d(C-A-A)]. Any manipulation of the poly[d(T-T-G)•d(C-A-A)] to reduce clc DNA, such as in producing B from A, further decreased its effectiveness.

(iii) Occurrence and detection of incorrect sequences arising during copying of poly[d(T-T-G)•d(C-A-A)]

A major problem in the production of non-clc poly[d(T-T-G)•d(C-A-A)] was the introduction of incorrect deoxynucleotide sequences into the product. The primary source of these wrong sequences appeared to be the oligodeoxynucleotide templates found in DIII or S factor preparations (Chapters IV and V). The addition of crude S

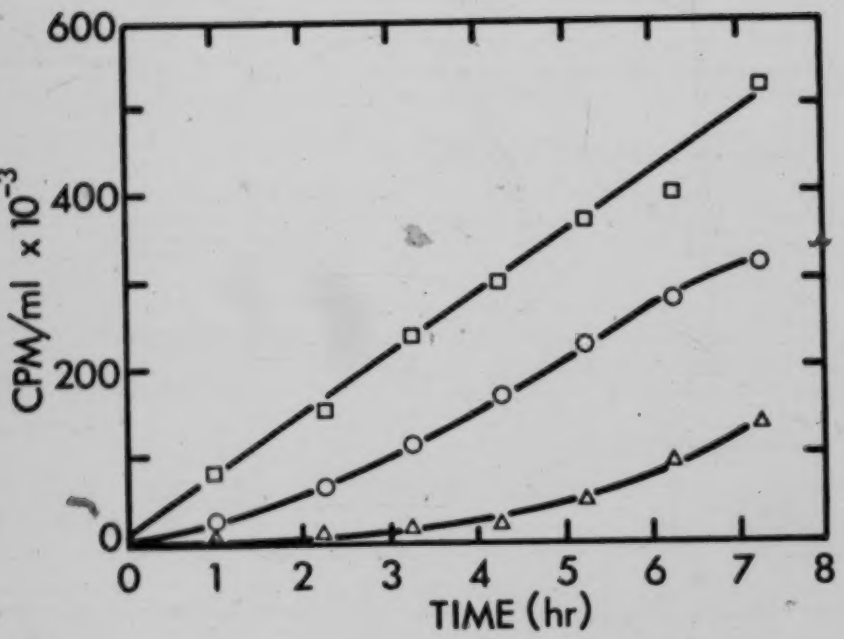


FIGURE 13. Comparison of Copying of Poly[d(T-G)•d(C-A)] and Poly[d(T-T-G)•d(C-A-A)]. The polymers were copied in the usual way with 0.5 ng/ml DNase I added to stimulate synthesis. The products were labelled with [^{3}H]dATP and [^{14}C]dCTP at specific activities of 1180 and 1350 cpm/nmole respectively. Synthesis was monitored by incorporation of label into TCA-insoluble material. Counts are given as open channel cpm. Squares, poly[d(T-G)•d(C-A)]; circles, polymer A; triangles, polymer B.

factor fractions resulted in extensive poly[d(A-T)•d(A-T)] synthesis which was detectable by the same means described for poly[d(T-G)•d(C-A)]. The addition of DIII resulted in the production of mixtures of poly[d(T-T-G)•d(C-A-A)] and either a poly[d(T,G)•d(C,A)] polymer (that is a polymer with random T and dG residues in one strand and dA and dC residues in the other), or a polypyrimidine•polypurine polymer similar to poly[d(T-C)•d(G-A)]. This was particularly a problem when the poly[d(T-T-G)•d(C-A-A)] had been partially degraded with endonuclease I before use as a template. Several methods were utilized to detect the presence of incorrect sequences:

a). Transcription by RNA polymerase. This technique was used to detect incorrect sequences and to establish the nucleotide ratios in the template polymers. Some typical results for authentic polymers and for samples contaminated by polymers of incorrect sequences are shown in Table V. The stimulation of rCTP incorporation by the addition of rUTP as seen for samples D and E (contaminated samples) was strongly indicative of the occurrence of dGMP and dAMP residues in the same strand. A similar argument can be made for rGTP and rATP. Transcription to poly[r(GTP)] when rGTP was the only nucleotide present, such as for D also indicated polypyrimidine•polypurine DNA content (Paetkau et al., 1972). The ratio of rCMP/rAMP incorporated was a useful indicator of anomalous sequences if it differed greatly

TABLE V

Detection of Incorrect Sequences by Transcription

[^{14}C]rNTP	Unlabelled rNTP	Incorporation (nmoles/ml) C[1]	D[1]	E[1]	F[1]	G[1]
rCTP	-	0	0	1.6	0	-
(2600 cpm/nmole)	rATP	3.3	0.6	0.8	6.5	-
	rUTP	0	13.6	10.3	0	-
	rGTP	0	0.5	1.3	0	-
rGTP	-	0	6.7	-	0	-
(2100 cpm/nmole)	rATP	0	11.3	-	-	-
	rUTP	6.4	1.7	-	-	-
	rCTP	0	3.2	-	-	-
rUTP	-	-	-	-	1.3	-
(2000 cpm/nmole)	rATP	-	-	-	1.3	-
	rGTP	-	-	-	19.7	-
	rCTP	-	-	-	1.3	-
Ratio rCMP/rAMP:	OBS:	0.47	-	0.44	0.84	0.90
	EXP:	0.5	0.5	0.5	0.5	1.0

[1]C: poly[d(T-T-G)•d(C-A-A)] copied from authentic poly[d(T-T-G)•d(C-A-A)].
D: poly[d(T-T-G)•d(C-A-A)] + polypyrimidine•polypurine contaminant.
E: poly[d(T-T-G)•d(C-A-A)] + polypyrimidine•polypurine contaminant.
F: poly[d(T-T-G)•d(C-A-A)] + poly[d(T,G)•d(C,A)] contaminant.
G: authentic poly[d(T-G)•d(C-A)].

from the expected value, as for preparation F. This ratio would not be expected to reveal incorrect sequences if there were sufficient correct sequences present to permit transcription with rCTP and rATP alone (preparation E).

b). Alkaline CsCl density gradient centrifugation. As stated earlier the segregation of ionizable bases into one strand of poly[d(T-T-G)•d(C-A-A)] leads one to expect that the strands would be separable in alkaline CsCl density gradients. Polymer D (contaminated with polypyrimidine•polypurine, Table V), despite having 2% clc DNA as measured by fluorescence, showed only one band in alkaline CsCl as seen in Figure 14a. Polymer F, with 8% clc DNA did in fact show 2 bands (Figure 14b), however, the densities were similar to those for poly[d(T-G)] and poly[d(C-A)] (Wells and Blair, 1967). Alkaline CsCl density gradient centrifugation of separable-stranded poly[d(T-T-G)•d(C-A-A)] was shown in Figures 11 and 12b.

c). Temperature-absorbance profile. The Tm provided a convenient means to detect poly[d(T,G)•d(C,A)] sequences in poly[d(T-T-G)•d(C-A-A)] preparations. The Tm's of various polymers were determined in 1/10 SSC as described in Chapter II. The results are shown in Table VI. Preparation F had a single sharp thermal transition and appeared to consist entirely of poly[d(T,G)•d(C,A)]. Preparation H showed 2

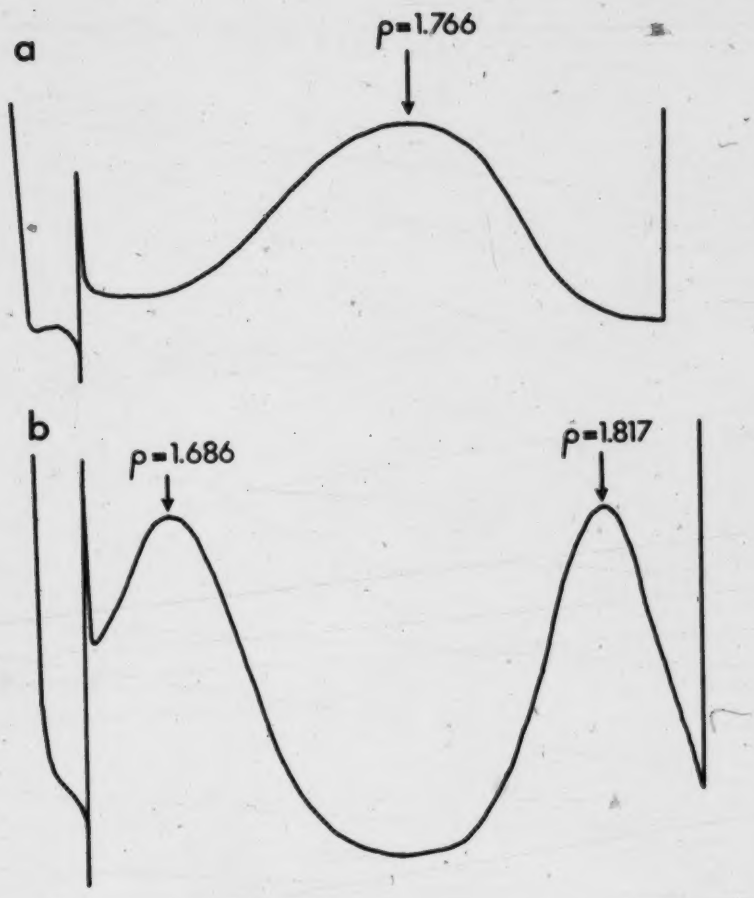


FIGURE 14. Analytical Alkaline CsCl Density Gradient Centrifugation of Samples of Polymers Containing Incorrect Sequences. The polymers were prepared in the presence of DIII and isolated. (a) preparation D, a product of poly[d(T-T-G)•d(C-A-A)] template pre-treated with endonuclease I before copying. (b) preparation F, a product of non-clc poly[d(T-T-G)•d(C-A-A)].

TABLE VI

Thermal Transition Temperatures for Various Poly[d(T-T-G)•d(C-A-A)] Preparations

Polymer Preparation	Tm (°C)	% Hyperchromicity
poly[d(T-G)•d(C-A)]	78°	32%
C [1]	70.5°	37%
F [1]	78°	30%
H [2]	70°	8.6% [3]
	78°	24%

[1] See Table V for designations of these preparations.
[2] This preparation appeared to be a mixture of poly[d(T,G)•d(C,A)] and poly[d(T-T-G)•d(C-A-A)].
[3] The total absorbance change over the two thermal transitions represented 34% hyperchromicity.

transitions. On the basis of hyperchromicity, H was estimated to contain 75% poly[d(T,G)•d(C,A)] sequences and 25% authentic poly[d(T-T-G)•d(C-A-A)]. Both F and H were prepared by copying an authentic poly[d(T-T-G)•d(C-A-A)] template.

Any polymer preparations suspected of containing undesirable sequences were tested by some or all of these methods. All these methods as well as the dCMP/dAMP ratio were used to characterize the non-clc poly[d(T-T-G)•d(C-A-A)] prepared as described earlier, to prove its authenticity.

The designation of the contaminant of some of the above preparations as poly[d(T,G)•d(C,A)] was made on the basis of several criteria. The resemblance to poly[d(T-G)•d(C-A)] was indicated by the stimulation of [^{14}C]rCTP incorporation by rATP during transcription, the rCMP/rAMP ratio, which approached 1, the sharp Tm at 78° and the banding of the separate strands in alkaline CsCl density gradients at the densities expected for poly[d(T-G)] and poly[d(C-A)]. No evidence was obtained as to the sequence in each strand, hence the designation as a random copolymer, poly[d(T,G)•d(C,A)].

III Discussion

Clc structures have been observed in three chemically defined polymers: poly[d(A-T)•d(A-T)] (a trivial case), poly[d(T-G)•d(C-A)], and poly[d(T-T-G)•d(C-A-A)]. Their presence has been detected by two separate procedures: an ethidium bromide fluorescence assay for renaturable DNA and alkaline CsCl density gradient centrifugation. Clc sequences accumulate during the copying of non-clc poly[d(T-G)•d(C-A)] or poly[d(T-T-G)•d(C-A-A)] by *E. coli* DNA polymerase I unless a source of S factor, such as DIII, is present. The copying of DNA polymers already containing clc sequences always leads to the production of more clc DNA despite the addition of a source of S factor. For this reason special means were required to obtain a non-clc poly[d(T-T-G)•d(C-A-A)].

Clc DNA production has been observed in other *in vitro* systems. *M. luteus* DNA polymerase produces clc poly[d(T-G)•d(C-A)] (Harwood and Wells, 1970). Schildkraut *et al.*, (1964) found non-denaturable and branched structures in natural DNAs synthesized *in vitro*. By means of the fluorescence assay it has been observed that the product of *in vitro* copying of *E. coli* DNA contains 100% clc DNA whereas the template had 0% clc DNA (Coulter *et al.*, 1974).

Polypyrimidine•polypurine DNAs such as poly[d(T-C-C)•d(G-G-A)], poly[d(T-C)•d(G-A)], and poly[d(A)•d(T)] have

never been demonstrated to contain clc sequences (Coulter et al. , 1974; Morgan et al. , 1974; Wells and Blair, 1967) either by the fluorescence assay or by CsCl density gradients. The physicochemical properties, including x-ray patterns, of the polypyrimidine•polypurine polymers indicate that they are structurally different from natural DNA (Wells et al. , 1970). The structural differences may cause a change in the copying mechanism reflected in the absence of clc structures.

The two methods used here to detect clc sequences - the ethidium bromide fluorescence assay and alkaline CsCl density gradients - do not necessarily yield the same quantitative results. The fluorescence assay will detect only material which has renatured and is therefore double-stranded. Any excess single-stranded segments are not included even though they may be covalently attached to the double-stranded region. However, such single-stranded segments will be included with the clc DNA in an alkaline CsCl density gradient unless the segments are extensive enough to shift the density towards that of the single strands.

Another means of detecting clc DNA in defined polymers is by nearest neighbour analysis. Polymers containing clc DNA have a significantly higher percentage of "wrong" nearest neighbours to dAMP (Coulter et al. , 1974). Clc DNA in natural DNAs may be detected by the ethidium bromide

fluorescence method or by hydroxyapatite chromatography.

The use of poly[d(T-G)•d(C-A)] and poly[d(T-T-G)•d(C-A-A)] as templates for further synthesis has been considered. There are few problems with poly[d(T-G)•d(C-A)]. With poly[d(T-T-G)•d(C-A-A)] there is a risk of introducing anomalous sequences especially if correct copying is proceeding slowly. This is in accordance with the findings of Wells et al., (1967). The increased complexity of a repeating tri- compared to a repeating dinucleotide would be expected to decrease its effectiveness as a template. The slippage mode (Chapter I) may play a smaller role in copying the repeating trinucleotide. Other templates present as contaminants of components of the synthesis mixture, as in DIII or S factor, may then be copied as well as, or more effectively than, the desired template. This would give rise to the polypyrimidine•polypurine, poly[d(T,G)•d(C,A)], or poly[d(A-T)•d(A-T)] polymers that have been observed. The solution to the problem was to use a highly purified S factor, free of poly[d(A-T)•d(A-T)] (Chapter II), rather than DIII in the copying of non-clc poly[d(T-T-G)•d(C-A-A)].

Although the problems with poly[d(T-T-G)•d(C-A-A)] were eventually overcome, the poor yields of non-clc templates and their inefficiency as templates for subsequent copying eliminates them as candidates for routine uses such as in assays for S factor activity.

Several structures are possible for clc DNA. Renaturable DNA copied from natural templates showed extensive branching (Schildkraut et al., 1964). Hairpin structures occur in poly[d(A-T)•d(A-T)] (Scheffler et al., 1968). The ratio (1:2) observed for single- to double-stranded molecular weights (Table IIIa) makes such structures unlikely for poly[d(T-G)•d(C-A)]. A branched structure may exist for clc poly[d(T-G)•d(C-A)].

Possible mechanisms by which clc structures arise involve either the polymerase switching strands or doubling back to copy the new strand. These mechanisms are illustrated in Chapter IV (Figure 22).

CHAPTER IV

PURIFICATION AND PROPERTIES OF S FACTOR

I Introduction

Extracts of *E. coli* contain a protein factor which prevents the accumulation of covalently linked complementary (clc) strands during the *in vitro* copying of poly[d(T-G)•d(C-A)] by *E. coli* DNA polymerase I. This protein was first observed (Paetkau, 1969) in Fraction IV from an *E. coli* DNA polymerase preparation (Richardson *et al.*, 1964b). A similar activity has also been found in DIII (Coulter *et al.*, 1974), a fraction from an *E. coli* RNA polymerase preparation (Chapter II). The protein has been designated "S" for "separability" (Coulter *et al.*, 1974). The purification from Fraction 4 and some of the properties of the S protein have been described (Flintoff, 1973; Flintoff and Paetkau, 1974).

This chapter describes a modification of the previous purification procedure, problems associated with the purification, and further characterization of the protein.

II Results: Purification of S Factor

A. Purification Procedure

Except for some minor modifications at the autolysis step, the procedure up to Fraction 6 was essentially the same as that described by Flintoff and Paetkau (1974). Therefore these steps are presented in outline only. The buffers are given the same designations, A-F, as in the original method. All procedures were carried out at 0-4° unless specified otherwise. The assay for S factor activity is described in Chapter II.

(i) Fraction 5

Fraction 4 was prepared according to the DNA polymerase purification of Richardson et al. (1964b) and included grinding of cells, streptomycin precipitation, autolysis, and ammonium sulfate fractionation. Fraction 5 was obtained from Fraction 4 by chromatography on DEAE-cellulose equilibrated with buffer A (0.2 M potassium phosphate (pH 6.5) - 10 mM 2-mercaptoethanol - 1mM EDTA). High molecular weight nucleic acids are adsorbed to such a column but DNA polymerase and S factor are not.

(ii) Fraction 6

Fraction 5 was chromatographed on G-25 Sephadex to remove low molecular weight nucleotidic material and salts and then concentrated fivefold by ultrafiltration in an Amicon cell (PM10 membrane).

An autolysis was then used to break down the high molecular weight complex with which S factor was associated. The concentrated material, still in buffer A, was warmed to 30° and magnesium chloride was added to 4 mM. Autolysis was monitored by the acid-solubilization of 0.5 A260 [^{14}C-dC,^{3}H-T]poly[d(T-G)•d(C-A)] (120,000 cpm/A260 unit) in an aliquot removed from the main portion of the material at the time of magnesium addition and incubated under the same conditions. The degradation of the labelled DNA proceeded with second order kinetics. The autolysis was stopped by cooling when extrapolation of a plot of the log of the percentage of undegraded DNA versus time indicated there should be less than 1% remaining. The time required varied between 120 and 180 minutes for different preparations.

The cooled sample was centrifuged 10 minutes at 10,000xg and applied by gravity to a G-75 Sephadex column (2.5x95 cm) equilibrated with buffer A. The elution profile is shown in Figure 15. S factor and a putative template for poly[d(A-T)•d(A-T)] were eluted together at a partially included position at 50% of the column volume. This

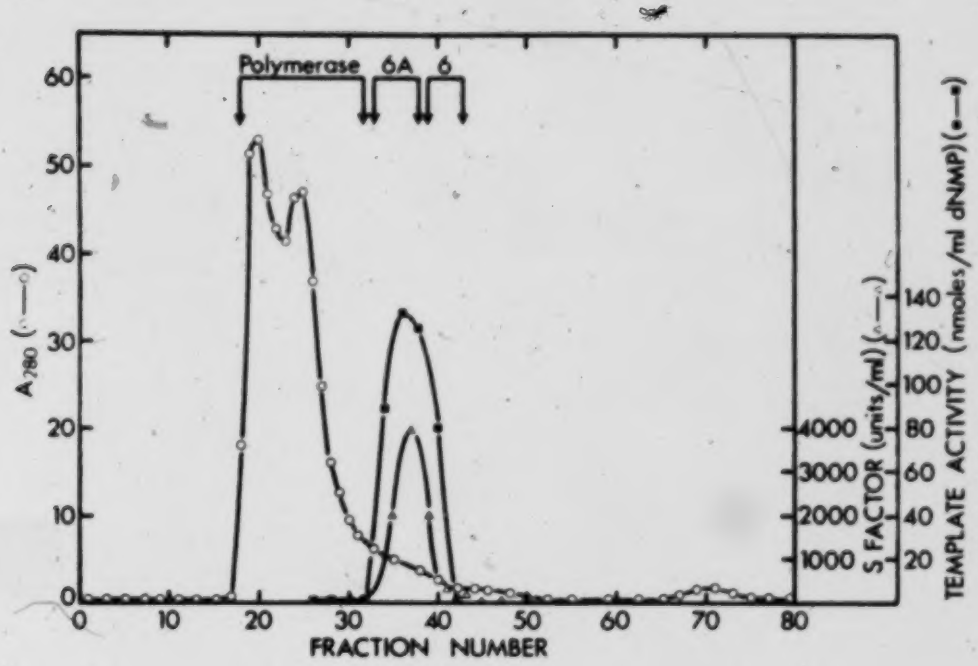


FIGURE 15. G-75 Sephadex Chromatography of S Factor Fraction 5. Autolyzed Fraction 5 (6 ml) was applied to a G-75 Sephadex column (2.5x95 cm) equilibrated with buffer A. The flow rate was 15 ml/hour and 6.4 ml fractions were collected. S factor and template activities were determined in the usual way.

position, relative to marker proteins of ovalbumin and chymotrypsinogen A in a separate run, was the same as that found by Flintoff and Paetkau (1974) and corresponded to a molecular weight of 26,000 for S factor. The material indicated as Fraction 6A in Figure 15 was used for some of the studies described in Chapter V but was not applicable for S factor purification because of its high template activity. The material designated as Fraction 6 was pooled (28 ml) and desalted by exclusion from a G-25 Sephadex column (2.5x38 cm) equilibrated with buffer B (10 mM Tris-Cl (pH 8) - 0.1 mM EDTA). The protein was lyophilized to dryness and dissolved in 2 ml of water to yield Fraction 6.

(iii) Fraction 7

The urea-LiCl treatment used to separate the S factor from a poly[d(A-T)•d(A-T)] template activity (Flintoff and Paetkau, 1974) was omitted for reasons to be discussed later (section C).

(iv) Fraction 8

DEAE-cellulose chromatography was used to reduce the nuclease content of Fraction 6. This procedure was similar to that described by Flintoff and Paetkau (1974). The elution profile for this column is shown in Figure 16. S factor activity was eluted between 0.10 and 0.23 M NaCl. Nuclease activity appeared in two areas: with unadsorbed

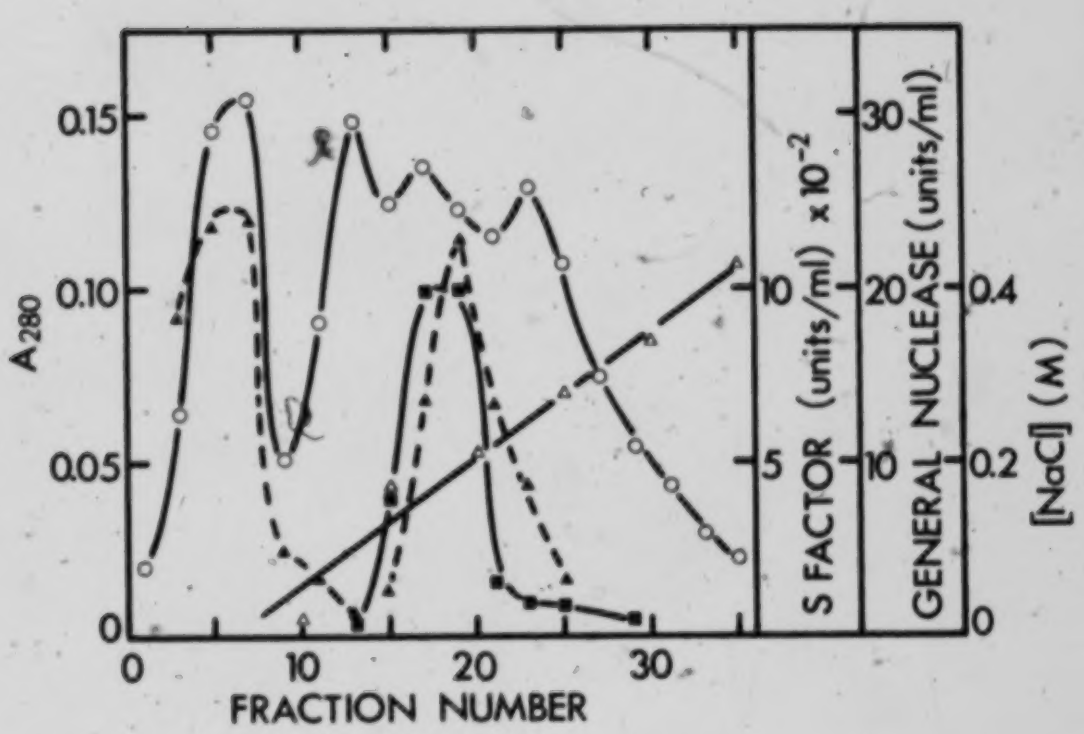


FIGURE 16. DEAE-Cellulose Chromatography of Fraction 6. One ml of Fraction 6 diluted with 4 ml buffer F (50 mM Tris-Cl (pH 8) 10% glycerol) was applied to a column (0.5x7 cm) equilibrated with buffer F. The column was washed with 2.5 ml buffer F, followed by a 30 ml linear gradient (15 ml buffer F, 0.03 M in NaCl; 15 ml buffer F, 0.45 M in NaCl) at a flow rate of 7.2 ml/hour. S factor and nonspecific nuclease activity were assayed in the usual way. Open circles, A280; squares, S factor activity; open triangles, [NaCl]; closed triangles, nuclease activity.

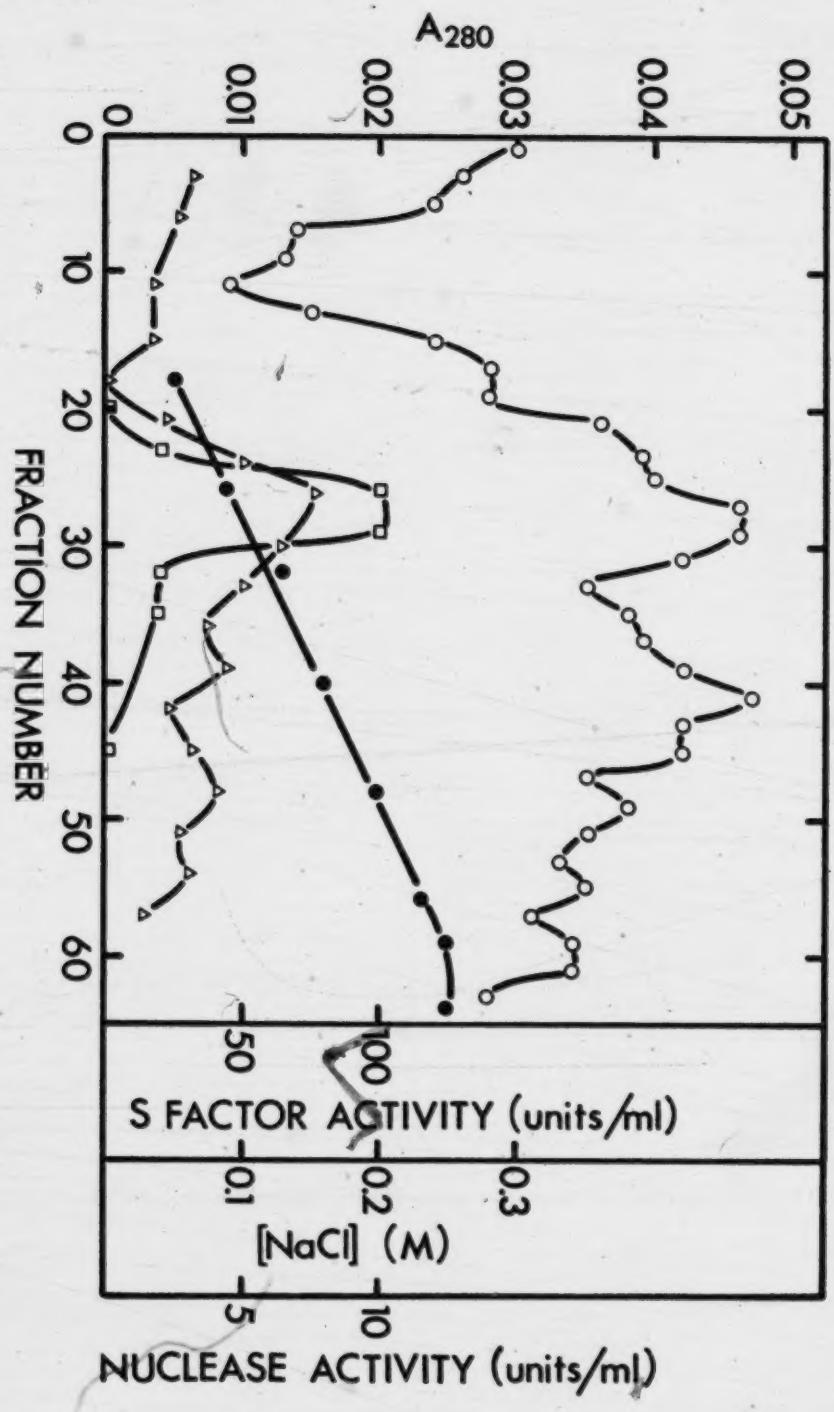
protein and between 0.13 and 0.20 M NaCl. The second nuclease peak was not resolved from S factor activity. Fractions containing S factor were pooled (6.5 ml) and desalted on a G-25 Sephadex column equilibrated with buffer B. The excluded material was concentrated by lyophilization and dissolved in 1 ml of water to yield Fraction 8.

The use of a larger column (1x7 cm) and gradient (150 ml) permitted chromatography of larger amounts of Fraction 6 at one time. However, the larger column resulted in 30-40% loss of S factor activity because of spreading out of the activity over a greater proportion of the gradient.

(v) Fraction 9

Residual nuclease activity was further reduced by chromatography on DEAE-Sephadex A25. The elution profile is shown in Figure 17. The main peak of S factor was eluted between 0.1 and 0.15 M NaCl. There was a considerable amount of spreading of S factor acitivity into the higher salt range of the gradient. Nuclease activity was reduced but not eliminated. Fractions containing S factor were pooled (16 ml) and desalted on a G-25 Sephadex column (2.5x38 cm) equilibrated with buffer B. The excluded material was concentrated by lyophilization and redissolved in 1 ml of water to give Fraction 9.

FIGURE 17. DEAE-Sephadex Chromatography of Fraction 8. Two ml of Fraction 8 were diluted with 8 ml buffer F and applied by gravity to a DEAE-Sephadex column (1x5 cm) equilibrated with buffer F. The column was washed with 10 ml buffer F followed by a 100 ml linear gradient (50 ml buffer F, 0.05 M in NaCl; 50 ml buffer F, 0.25 M in NaCl) at a flow rate of 12 ml/hour. S factor and nuclease activities were assayed in the usual way. Open circle, A280; squares, S factor activity; closed circles, [NaCl]; triangles, nuclease activity.


A280
0
0.01
0.02
0.03
0.04
0.05
FRACTION NUMBER
0
10
20
30
40
50
60
S FACTOR ACTIVITY (units/ml)
50
100
[NaCl] (M)
0.1
0.2
0.3
NUCLEASE ACTIVITY (units/ml)
5
10

B. Characterization of S Factor Fractions

This section summarizes various characteristics of the S factor fractions. All of the data refer to the same preparation described in Table VII unless otherwise indicated.

(i) S factor activity

A summary of the yields of protein and S factor activity for each fraction is shown in Table VII. The low yield of S factor activity in Fraction 6 may not be real since high levels of nuclease activity in Fractions 4 and 5 tend to mimic S factor activity as measured by the fluorescence assay.

Titration curves of Fraction 6,8, and 9 (Figure 18a) show the effect of increasing amounts of the various fractions on the percent clc DNA formed during copying of poly[d(T-G)•d(C-A)]. The relationship between the amount of S factor added and the reduction in clc DNA is not a simple one. The increase in the percent clc DNA at high levels of S factor is due to the production of poly[d(A-T)•d(A-T)] as will be discussed below. Where this occurs, extrapolation must be used to calculate the number of units/ml of S factor activity (Chapter II). Fractions 4 and 5 do not generally show this inflection. This is probably the result of the high nuclease activity which tends to destroy poly[d(A-

TABLE VII

Purification of S Factor[1]

Fraction	Total Protein (mg)	Total Activity (units)	Specific Activity (units/mg)	Yield[4] (%)
4 Ammonium Sulfate	800[2]	60,000	75	100
5 DEAE-Cellulose I	480[2]	48,000	100	80
6 G-75 Sephadex	7.5[3]	10,000	133	16.7
8 DEAE-Cellulose II	1.0[3]	10,000	10,000	16.7
9 DEAE-Sephadex	0.28[3]	600	2120	1

[1]From 400 g *E. coli* cells.
[2]Determined by the Biuret method (Chapter II).
[3]Determined by A280.
[4]Based on units present in Fraction 4.

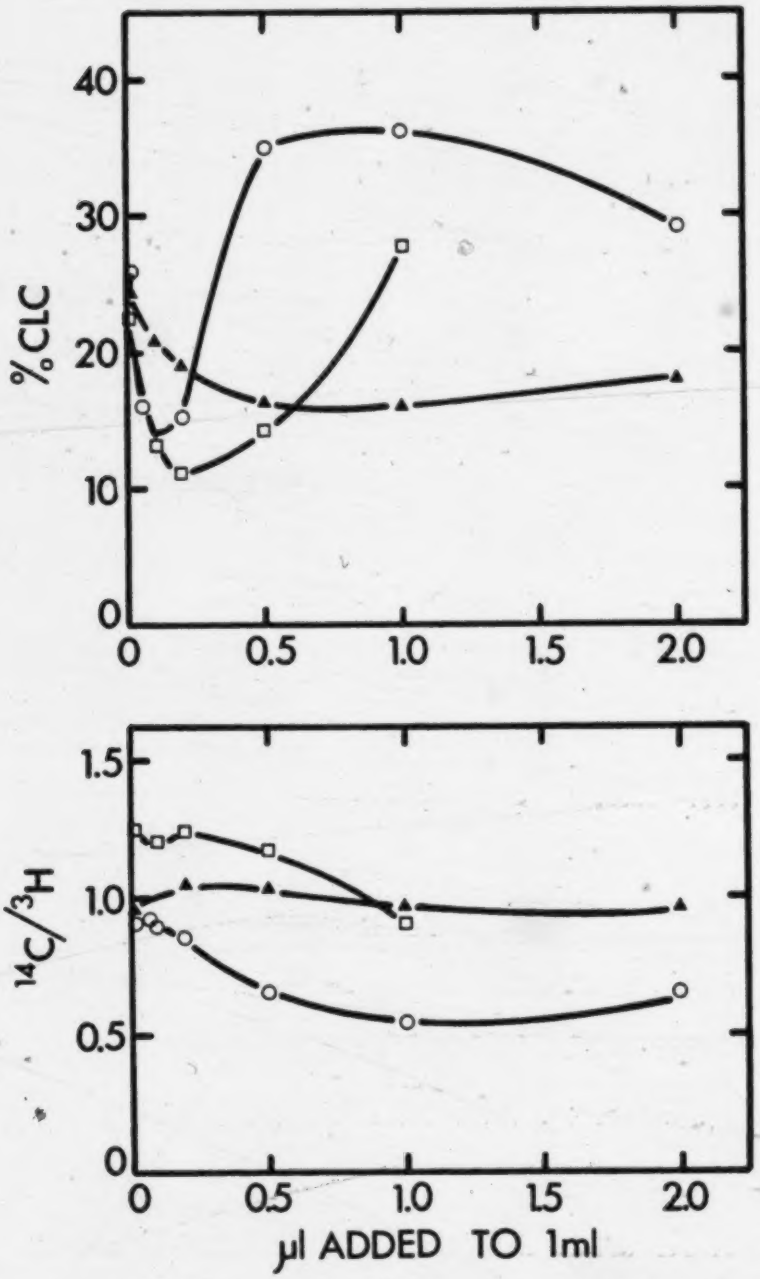


FIGURE 18. Titration Curves of S Factor Activity in Fractions 6, 8, and 9. Various dilutions of the fractions were assayed for S factor activity during poly[d(T-G)•d(C-A)] synthesis. (a) % clc DNA measured by ethidium bromide fluorescence. (b) [^{14}C]dCMP/[^{3}H]TMP incorporated into TCA-insoluble material and counted in restricted isotope channels correcting for overlap. Circles, Fraction 6; squares, Fraction 8; triangles, Fraction 9.

T)•d(A-T)] as it is made.

(ii) Template activity for poly[d(A-T)•d(A-T)]

The presence of a template for poly[d(A-T)•d(A-T)] in Fractions 6 and 8 is suggested by the increase in percent clc DNA (Figure 18a) and by the decrease in the ratio of incorporated [^{14}C]dCMP/[^{3}H]TMP (Figure 18b) at critical concentrations of these fractions. Fraction 9 did not show these changes so clearly but the rather high plateau value for percent clc DNA may be due to production of low levels of poly[d(A-T)•d(A-T)]. A specific assay for poly[d(A-T)•d(A-T)] synthesis from Fraction 9 showed that the template was still present (Chapter V).

(iii) Nuclease activity

Table VIII presents a summary of the nuclease activities found in the various fractions. The two substrates used gave approximately the same results in the absence of tRNA. Poly[d(A-T)•d(A-T)] is slightly more sensitive to nucleolytic attack than is poly[d(T-G)•d(C-A)]. The nuclease activity inhibited by tRNA was assumed to be endonuclease I on the basis of the known effect of tRNA on this nuclease (Lehman et al., 1962).

The high nuclease acitivity of Fraction 6 was likely responsible for the decrease in percent clc DNA at very high

TABLE VIII

Nuclease Activities in S Factor Fraction[1]

Fraction	Nonspecific Nuclease[2] Total Units	tRNA Inhibitable Nuclease[3] Total Units -tRNA	+tRNA	% Inhibition
6	-	1200	560	53%
8	59.2	-	-	-
9	23.0	16.4	15.1	8%

[1] Assays described in Chapter II.
[2] poly[d(A-T)•d(A-T)] substrate.
[3] poly[d(T-G)•d(C-A)] substrate.

concentrations of Fraction 6 (Figure 18a). The nuclease content was reduced by the DEAE-celluose and DEAE-Sephadex chromatographies. One preparation of Fraction 9 showed no nuclease activity measurable by the usual assays. This preparation was used where indicated.

(iv) Protein components of S factor fractions

The protein components, their molecular weights, and approximate percentage of the total composition were determined by SDS gel electrophoresis using 10% polyacrylamide gels (Chapter II). Tracings of the gels for Fractions 6, 8, and 9 are seen in Figure 19. An analysis of the tracings in terms of the molecular weights of the components and their percent of the total composition is shown in Table IX.

The molecular weight determined for S factor is in agreement with that observed by Flintoff and Paetkau (1974). Fraction 9 represents a highly purified S factor.

(v) Stability

Fractions 6 to 9 were stable for at least several months when stored on ice. Nuclease activity in Fraction 6 decreases slightly with storage.

FIGURE 19. SDS Polyacrylamide Gel Electrophoresis of S Factor Fractions. SDS polyacrylamide (10%) gels were run, stained with Coomassie Brilliant Blue, and traced as described in Chapter II. (a) Fraction 6, 7.5 ug; (b) Fraction 8, 5 ug; (c) Fraction 9, 3.5 ug. Further analysis of the indicated components is shown in Table IX.

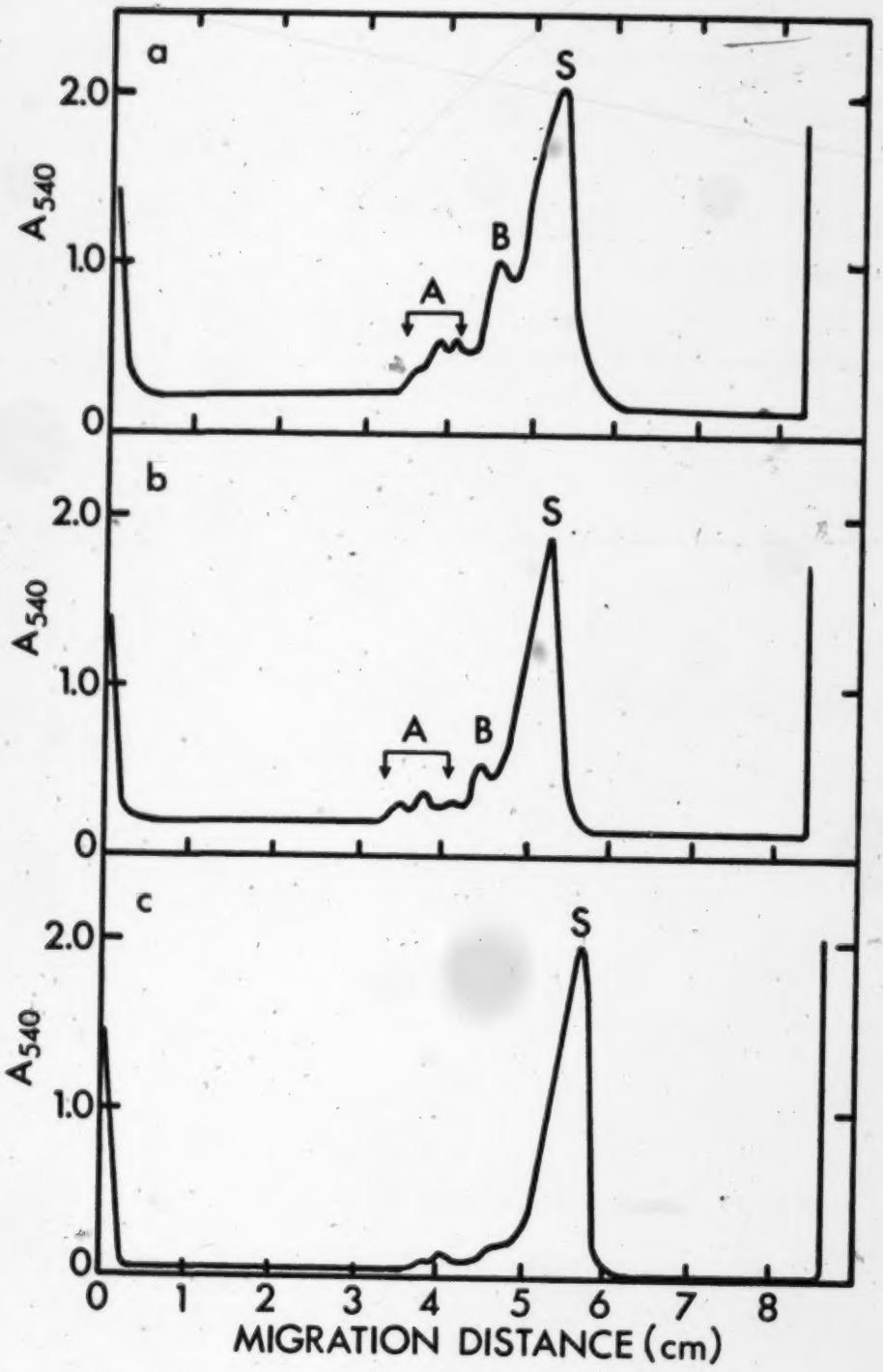

a
b
c
S
A
B
2.0
1.0
0
A_{540}
0 1 2 3 4 5 6 7 8
MIGRATION DISTANCE (cm)

TABLE IX

Protein Components of S Factor Fractions

Fraction	Components[1]	Molecular Weight[2]	Percent of Total Composition[3]
6	A	23,000-26,000	12±2%
	B	18,500	20±5%
	S	11,500±500	70±5%
8	A	26,000-29,000	9±2%
	B	19,500	15±4%
	S	11,500±1000	75±4%
9	S	10,000±1000	85±5%
9 [4]	S	9500±1000	94±5%

[1] As indicated in Figure 19.
[2] Determined by comparison to standard proteins.
[3] Determined by comparison of areas under peaks in tracings.
[4] Tracing not shown. Nuclease-free preparation.

C. Problems in Purification of S Factor

As was indicated in the previous section, S factor is associated with a putative template for poly[d(A-T)•d(A-T)]. This agrees with the findings of Flintoff and Paetkau (1974). It will be shown later (Chapter V) that the agent responsible for the template activity appears to be an oligodeoxynucleotide. The major difficulty in the purification of S factor is the removal of this template.

(i) Denaturation with combinations of urea, LiCl, and CsCl

The removal of the template has been accomplished in a limited number of cases using a method adapted from Traub and Nomura (1968). Briefly, this method consisted of a 36 hour incubation of Fraction 6 at 0° in 4 M urea - 4 M LiCl - 6 mM 2-mercaptoethanol followed by chromatography on a G-50 Sephadex column equilibrated with buffer C (4 M urea - 4 M LiCl - 10 mM Tris-Cl (pH 7.5) - 6 mM 2-mercaptoethanol). The excluded material, containing S factor, was dialyzed to remove the urea and LiCl. The resultant Fraction 7 no longer possessed the template activity but had lost about 50% of its S factor activity. This procedure was not effective in the present study except when performed twice on the same material, in which case it was accompanied by a 90% loss of S factor activity.

Variations on this procedure were used in an attempt to improve its effectiveness. The use of the above conditions with 30% CsCl present, 4 M urea alone or 4 M LiCl alone did not achieve the removal of the template activity.

A urea-LiCl-CsCl density gradient was used in an attempt to effect a separation of S factor and the template activity. Fraction 6 (700 ug) in 0.2 M Tris-Cl (pH 8) - 2 mM EDTA was incubated for 42 hours at 0° with 4 M urea - 4 M LiCl. This sample (0.4 ml) was then layered onto 4.9 ml of a solution of buffer C containing 0.1 mM EDTA and 36.2 g% CsCl. The initial density of the solution was 1.4 g/cm³ determined pycnometrically. The sample was centrifuged as shown in Figure 20. Fractions from the gradient were tested for S factor and poly[d(A-T)•d(A-T)] template by the usual methods. The two activities banded together at the top of the gradient. In an identical separate experiment, two markers, chymotrypsinogen A (5 mg) and [^{3}H-T]poly[d(A-T)•d(A-T)] (0.16 A260 units) banded separately (Figure 20b). The density difference between protein at 1.4 g/cm³ (Weigle et al., 1959) and poly[d(A-T)•d(A-T)] at 1.672 g/cm³ (Wells and Blair, 1967) in neutral CsCl without urea and LiCl indicated that protein and DNA should separate unless they are firmly associated. This was found in the case of authentic high molecular weight poly[d(A-T)•d(A-T)] and chymotrypsinogen A. The addition of urea and LiCl was not sufficient to dissociate S factor and the template.

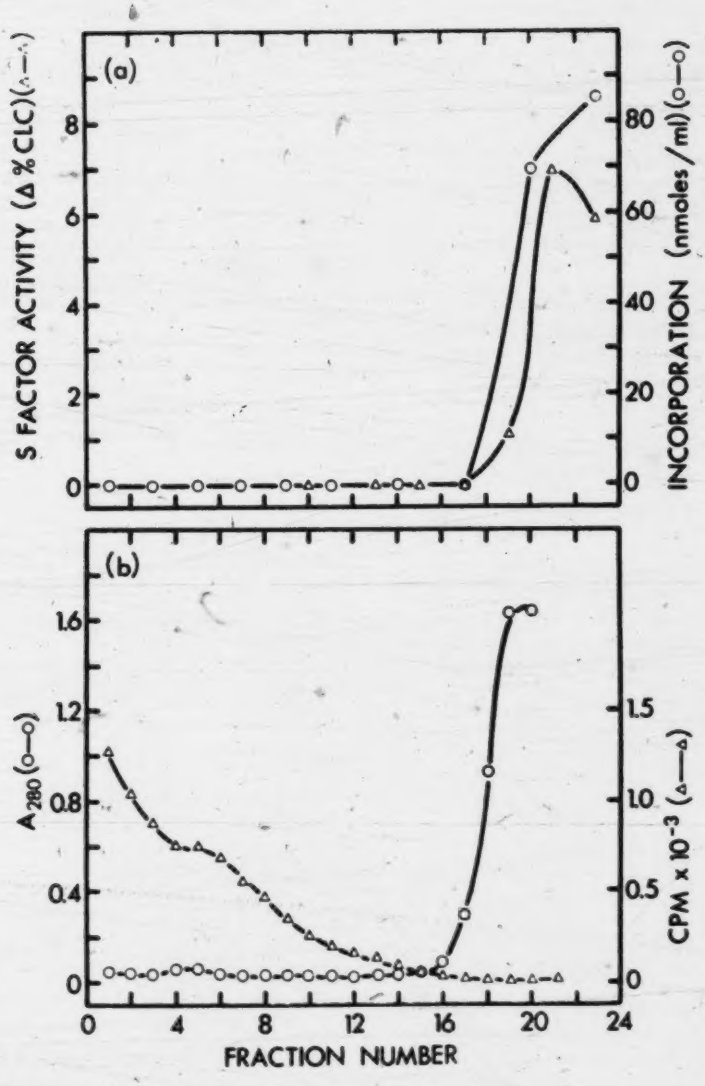


FIGURE 20. Urea-LiCl-CsCl Density Gradient Centrifugation. Samples of Fraction 6 (a) and markers (b) were prepared as given in the text and centrifuged in an SW 65L Ti rotor for 35 hours at 50,000 rpm at 5°. Fractions (0.2 ml) were pumped from the bottoms of the tubes. (a) Fraction 6: circles, template activity; triangles, S factor activity given as a decrease in percent clc compared to a control with no S factor. (b) markers; triangles, TCA-insoluble [^{3}H-T]poly[d(A-T)•d(A-T)]; circles, A280 of chymotrypsinogen A.

(ii) SDS or Sarkosyl treatment

Both SDS and Sarkosyl tend to disrupt nucleic acid-protein associations and are used routinely for this purpose in the isolation of DNA polymers after polymerase-mediated synthesis.

Two samples of Fraction 5 were diluted to 9 mg/ml of protein in buffer G (20 mM Tris-Cl (pH 7.5) - 10 mM 2-mercaptoethanol - 1 mM EDTA). Samples 1 and 2 were made 3% in SDS and Sarkosyl respectively. Both samples were incubated at 37° for 10 minutes, then applied to G-25 Sephadex columns (0.9x16 cm) equilibrated with buffer G. The excluded fractions were tested for S factor and template activities with the results shown in Table X. There was approximately 60% recovery of S factor in the excluded material for both samples. The template activity and S factor remained associated.

(iii) Phenol extraction

Phenol has been a useful tool in the extraction of nucleic acids (Kirby, 1968) without being destructive to small proteins (Rushizky et al., 1963).

Three samples, each containing 30 mg/ml Fraction 5 in buffer G were treated with combinations of phenol and SDS. Sample 3 had no SDS added. Samples 4 and 5 were made 1% in SDS and heated 10 minutes at 37° and 80° respectively. Each

TABLE X

Extraction of Poly[d(A-T)•d(A-T)] from S Factor

Sample	Dilution into Assay	Template Activity[1]	S Factor Activity[2]	% Recovery
1 (SDS)	1/500 1/10,000	47 [3]	11	60%
2 (Sarkosyl)	1/500 1/300	242 [3]	11	60%
3 (phenol)	1/500 1/1000	207 [5]	2	<10%
4 (phenol + SDS, 37°)	1/500 1/1000	167 [5]	9	30%
5 (phenol + SDS, 80°)	1/500 1/1000	210 [5]	9	30%
Fraction 5 (Untreated)	1/500 1/10,000	47 [3]	6	-

[1] Incorporation of [^{3}H]TMP in nmoles/ml.
[2] Decrease in percent clc DNA compared to control.
[3] Incorporation during 7 hours.
[4] Incorporation during 2 hours.
[5] Incorporation during 6 hours.

sample was extracted twice with one volume of phenol saturated with buffer G. The combined phenol phases were extracted with one volume of buffer G. The phenolic material was then lyophilized until no more phenol odour was detectable. Each sample was dissolved in 0.5 ml of water and applied to G-25 Sephadex columns (0.9x16 cm) equilibrated with buffer G. The excluded material from each column was lyophilized, dissolved in 0.2 ml water, and tested for S factor and template activities. The results are shown in Table X. There was about 30% recovery of S factor activity for samples 4 and 5. A large amount of material from sample 3 was lost in an insoluble precipitate formed after lyophilization of the phenol extract. S factor and template activities remained associated in all cases.

(iv) Enzymatic degradation of template activity

The template activity was not susceptible to nucleolytic degradation by endogenous nucleases, exonuclease III, or a combination of pancreatic DNase I and venom phosphodiesterase unless the accompanying proteins were first denatured. This will be discussed more fully in Chapter V.

These results show that S factor and the oligo[d(A-T)•d(A-T)] template are not readily separable by nondestructive denaturing conditions without extensive

losses of S factor. For this reason the removal of the template was not performed and purification to Fractions 8 and 9 was done omitting Fraction 7. With Fraction 9, the interference with S factor assays by poly[d(A-T)•d(A-T)] production could be decreased by sufficient dilution. However, as will be shown in Chapter V, the template is still present.

III Results: Properties of S Factor

A. Effect Of S Factor on clc DNA Production in vitro

(i) Copying of chemically defined DNA polymers

The prevention of accumulation of clc structures during the copying of poly[d(T-G)•d(C-A)] forms the basis of the assay for S factor and was its initial defining characteristic. Poly[d(T-G)•d(C-A)] made in the absence of S factor normally has about 25% clc DNA as measured by the fluorescence assay. The addition of S factor reduces the percent clc DNA (Figure 18). The product polymer has separable strands. A similar effect has been observed for poly[d(T-G-G)•d(C-A-A)] (Chapter III, Figure 12). The difference between clc and non-clc polymers is seen clearly in alkaline CsCl density gradients.

S factor is unable to remove covalent links once they have formed either when it is added after copying of poly[d(T-G)•d(C-A)] has begun or upon incubation with clc

poly[d(T-G)•d(C-A)] (Flintoff and Paetkau, 1974).

(ii) Copying of bacteriophage PM2 DNA

PM2 RFII DNA was copied under the conditions described in Chapter II. One synthesis mixture contained no S factor and the other contained 1 ug/ml Fraction 9 (nuclease free). The products were labelled with [^{14}C]dCTP (1415 cpm/nmole) throughout synthesis. At 3.5 hours (1.5 rounds of copying) [^{3}H]TTP was added to give a specific activity of 12,000 cpm/nmole TTP. The synthesis was stopped at 6 hours by the addition of EDTA to 25 mM and cooling. The products were examined by three different means:

a). Ethidium bromide fluorescence. Aliquots removed at intervals during the copying were diluted into 3 ml of KE buffer for the fluorescence assay (Chapter II). The results given in Figure 21 show that S factor did not entirely prevent clc DNA production. In the early stages of the synthesis, the percent clc DNA was very high and then decreased to a plateau level of 60% (Figure 21a). Non-clc DNA increased steadily after one round of copying in the presence of S factor (Figure 21b). Without S factor, the production of non-clc DNA was much less. S factor stimulated synthesis by 75%.

b). Batchwise hydroxyapatite analysis. Hydroxyapatite chromatography was performed as described in Chapter II. Samples were divided in half and one half was

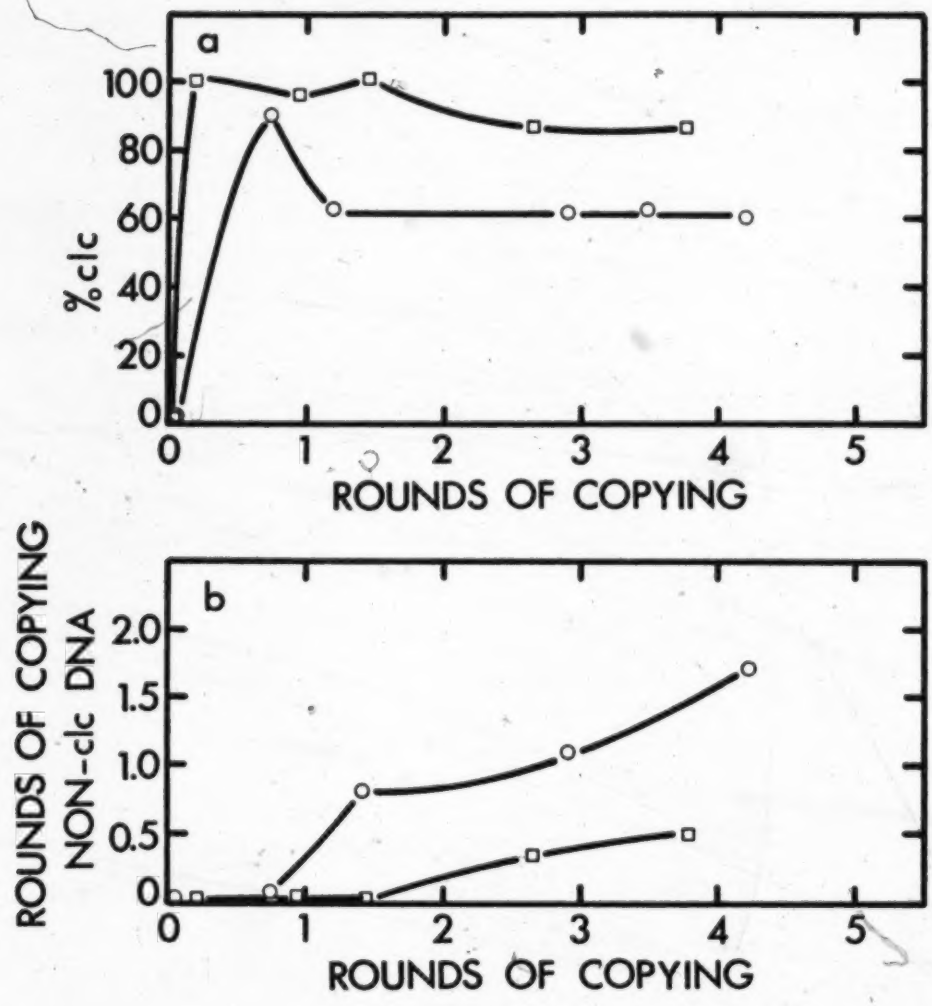


FIGURE 21. Clc DNA Production During in vitro Copying of PM2 RFII DNA. PM2 DNA was copied in the presence and absence of S factor as described in Chapter II. The fluorescence assay in the KE buffer system was used to monitor total and clc DNA production. The results are expressed as percent clc DNA versus rounds of copying (a), and rounds of copying non-clc DNA production versus rounds of copying total DNA (b). Circles, with S factor; squares, without S factor.

sonicated (5x2 seconds on optimal setting) and denatured by heating at 95° for 5 minutes then quickly cooled in ice before adding the hydroxyapatite. Sonication would be expected to decrease the proportion of single-stranded material attached to double-stranded DNA. After denaturation only clc DNA will renature and behave as double-stranded DNA on the hydroxyapatite. After each extraction aliquots from the supernatants were added to Aquasol and counted in restricted isotope channels. Synthesis in the presence of S factor increased the percentage of single strands (i.e. non-clc DNA) in ^{3}H-labelled material from 6.3% to 28% and in ^{14}C-labelled material from 8.9% to 25.3%. Sonication increased the proportion of single strands slightly. The proportion of ^{14}C-labelled single-stranded material was similar to that for ^{3}H-labelled material after synthesis in the presence of S factor, suggesting that the decreased renaturability was not due to a general nuclease effect. If this were the mechanism, then the ^{14}C-labelled DNA, which was exposed to S factor longer, would have had a greater proportion of single-stranded DNA than the ^{3}H-labelled material.

c). Alkaline sucrose sedimentation. Samples from 6 hours of synthesis were made 17 mM in NaOH and layered onto 5%-20% sucrose gradients containing 0.1 M NaCl - 10 mM EDTA - 0.10 M NaOH. The gradients were centrifuged in an SW 65L Ti rotor at 60,000 rpm for 3.5

hours at 5°. The DNA synthesized in the presence of S factor appeared to be of slightly lower molecular weight than that made without S factor. There was no separation of ^{3}H- and ^{14}C-labelled material.

These results indicate that S factor had an effect on the structure of the synthesized DNA. The results of the fluorescence assay and the Hydroxyapatite analysis suggest that clc DNA production was reduced. The decreased molecular weight of the product made in the presence of S factor may be due to a low level of nuclease activity as well as synthesis of short fragments along the displaced 5' strand (see Discussion, Figure 23). The similarity in the proportion of ^{3}H- and ^{14}C-labelled material seen in the hydroxyapatite analysis and the co-sedimentation of ^{3}H- and ^{14}C-labelled DNA suggest that the effect of S factor is specific and cannot be attributed to a general nuclease effect.

B. Proteolytic Effect of S Factor on DNA Polymerase

Limited proteolysis of DNA polymerase I produces the 76,000 molecular weight component which retains polymerase activity (Brutlag et al., 1969). S factor's activity therefore might be effected through some proteolytic alteration of the DNA polymerase. To test this possibility, DNA polymerase (70 ug/ml) was incubated with 10 ug/ml S factor (Fraction 9) or S factor plus the 4 dNTPs (1.2 mM each) and/or 0.2 A260 poly[d(T-G)•d(C-A)]. Potassium

phosphate, DTT, and magnesium chloride were used as in a normal synthesis mixture for poly[d(T-G)•d(C-A)]. After two hours at 37° the reaction was stopped by the addition of EDTA to 20 mM. The samples were then subjected to electrophoresis on SDS polyacrylamide (5%) gels for 1.5 hours. The major protein bands seen were the 109,000 and 76,000 molecular weight components of DNA polymerase (Chapter II) and S factor where it was added. In all cases, the ratio of 76,000/109,000 material was within 0.03 of the value obtained without S factor treatment (0.10). The ratio of the migration distance of the 109,000 component to that of S factor itself or to that of the 76,000 component varied less than 2%. These results suggested that there was neither an alteration in the molecular weight of the 109,000 component of DNA polymerase nor any specific conversion of the 109,000 component to the 76,000 component.

C. Effect of S Factor on Repair of Single-Stranded DNA

DNA polymerase I may take part in repair <u>in vivo</u> (Chapter I). The effect of S factor on repair was therefore tested using poly[d(T-G)] and poly[d(C-A)] templates and the decadeoxynucleotides oligo[d(C-A)] and oligo[d(T-G)] respectively as primers. The reaction mixture for repair synthesis consisted of 70 mM potassium phosphate (pH 7.4) - 10 mM magnesium chloride - 2 mM DTT - 0.12 mM each of TTP and dGTP or dCTP and dATP - 20 nmoles/ml template (poly[d(C-A)] or poly[d(T-G)] - 140 units/ml DNA polymerase. The

mixture was incubated at 15°. Repair synthesis was measured by the incorporation of [^{3}H]dCTP or [^{3}H]TTP at specific activities of 6225 and 7100 cpm/nmole respectively. The incorporation reached a plateau at 0.4- to 0.5-fold copying in 30 minutes. The repair synthesis using S factor at concentrations of 4, 2, 1, or 0 ug/ml varied less than 10% in rate and extent.

D. Binding of S Factor to Poly[d(A-T)•d(A-T)]

The firm association between S factor and poly[d(A-T)•d(A-T)] suggested that binding of S factor to high molecular weight poly[d(A-T)•d(A-T)] might be demonstrable. Three methods were used as indicators of binding:

a). Protection from exonuclease III action. The acid-solublization of poly[d(A-T)•d(A-T)] by exonuclease III was compared in the presence and absence of S factor (Fraction 9). S factor was present at concentrations giving 3 to 12 protein molecules per molecule of DNA. The presence of S factor did not decrease the rate or extent of degradation of the poly[d(A-T)•d(A-T)] by exonuclease III.

b). Ethidium bromide binding. The amount of ethidium bromide that can bind to DNA may be decreased by the prior binding of proteins to the DNA. Poly[d(A-T)•d(A-T)] was incubated at room temperature in 30 mM potassium phosphate (pH 7.4) with a 1- to 10-fold molar excess of S factor (Fraction 9). Variations included:

addition of magnesium chloride to 4 mM and/or DNA polymerase to 35 ug/ml; extending the incubation time to 12 hours; or replacing poly[d(A-T)•d(A-T)] with *E. coli* DNA. Samples were added to 3 ml of TEE buffer and the fluorescence measured within 10 minutes. The resultant fluorescence varied less than 5% from a control with no S factor.

c). Agarose chromatography. Tritium-labelled poly[d(A-T)•d(A-T)] (0.08 A260 units) and 20 ug S factor (Fraction 9) were incubated together in a 200 ul reaction mixture consisting of 0.15 M Tris-Cl (pH 8) - 1 mM EDTA. A parallel sample contained no DNA. After 1 hour at 4°, the samples were applied to 15M Agarose columns (0.9x30 cm) equilibrated with buffer B. Fractions were 1 ml. The excluded material was detected by the radioactivity of the poly[d(A-T)•d(A-T)]; the included material by absorbance of an ATP marker . The included and excluded fractions were pooled separately and lyophilized to dryness. The samples were taken up in 0.5 ml of water and dialyzed against 5 mM sodium phosphate (pH 7.2) at 4°. The dialyzed samples were concentrated by evaporation and subjected to SDS electrophoresis on 10% polyacrylamide gels (Chapter II). For both samples S factor was found only in the included material. It did not co-chromatograph with the poly[d(A-T)•d(A-T)].

Binding was not detectable by any of these methods.

IV Discussion

S factor has been purified to an essentially homogeneous protein, Fraction 9. It has a molecular weight of 9500-12,000 on SDS gel electrophoresis and an apparent molecular weight of 24,000-26,000 on G-75 Sephadex. These values agree with those reported by Flintoff and Paetkau (1974). It has been suggested that the protein may consist of a dimer of identical subunits. The protein has a template for poly[d(A-T)•d(A-T)] bound to it. It does not appear to bind to high molecular weight poly[d(A-T)•d(A-T)]. S factor does not have a proteolytic effect on DNA polymerase nor does it stimulate repair synthesis by DNA polymerase. S factor does, however, reduce or prevent clc DNA production during copying of the DNA polymers poly[d(T-G)•d(C-A)] and poly[d(T-T-G)•d(C-A-A)]. It appears to have a similar effect during <u>in vitro</u> copying of PM2 DNA.

There are a number of problems which complicate the purification. One of these is the lack of a simple relationship between the amount of S factor present and the decrease in the percent clc DNA. This introduces a degree of subjectivity into an estimation of activity. The presence of nuclease activity and a template for poly[d(A-T)•d(A-T)] also make these calculations more difficult. Nuclease activity can mimic S factor, and poly[d(A-T)•d(A-T)] copying can increase clc DNA.

The S factor activity measured in Fractions 4 and 5 should be considered as only approximate values because of the high levels of nuclease activity. Fraction 6 is more amenable to assay. The absolute yield of S factor activity in Fraction 6 is similar to that reported by Flintoff and Paetkau (1974). The addition of tRNA to the assay system increases the accuracy of the assay by inhibiting endonuclease I (Lehman et al., 1962). It was shown in Table VIII that the nuclease activity in Fraction 6 is 53% inhibitable by tRNA. It should be noted that endonuclease I does not mimic the effect of S factor when the synthesis products are examined by alkaline CsCl density gradients (Coulter et al., 1974).

The template for poly[d(A-T)•d(A-T)] bound to S factor masks the effectiveness of high concentrations of S factor. The titration curves in Figure 18 show that the concentration of S factor necessary to obtain a low level of clc DNA is very critical. The elimination of the template proved to be unpractical. The lack of success with phenol extraction and urea-LiCl or detergent denaturation followed by column chromatography could be explained on the basis of the template's size, causing it to be excluded along with the protein. The two would presumably then reassociate. That size alone is not the only contributing factor is indicated by co-banding of S factor and the template in a urea-LiCl-CsCl density gradient. The procedure decribed by Flintoff

and Paetkau (1974) (chromatography in 4 M LiCl - 4 M urea) was successful in some cases and there are several possible reasons for its lack of general effectiveness. The size of the template may vary from one preparation to another depending on the degree of autolysis. The procedure was effective only when it was accompanied by extensive losses of S factor activity so there may be a selection for S factor molecules which are free of template.

S factor and the template activities co-chromatograph at a partially included position on G-75 Sephadex following an autolysis at the Fraction 5 stage. Without the autolysis, the S factor is completely excluded along with DNA polymerase (Flintoff and Paetkau, 1974). The autolysis is dependent on the prior removal of low molecular weight components by G-25 Sephadex chromatography and the addition of magnesium. The change in the apparent molecular weight of the S factor during the autolysis is assumed to be due to partial degradation by endogenous nucleases of the DNA fragments to which the protein is bound. It might be argued that the molecular weight change is in the S protein itself. This cannot be ruled out but it is unlikely for two reasons: the autolysis is dependent on magnesium which is characteristic of nucleolytic not proteolytic degradation; and SDS gel electrophoresis of Fractions 4 and 5 showed proteins with molecular weights in the 9000-13,000 range, one of which could be S factor.

The association of S factor with a template for poly[d(A-T)•d(A-T)] suggests an affinity for such sequences. This may be related to the actual binding sites for the protein on template DNA during copying. The lack of observable binding to high molecular weight poly[d(A-T)•d(A-T)] may be a function of the size of the polymer. Binding to the ends of the polymer may not be detectable at the higher ratio of internal to terminal residues found in high molecular weight polymers compared with shorter polymers.

S factor does not stimulate repair synthesis nor does it behave as a protease towards DNA polymerase. S factor is distinguishable from other low molecular weight proteins such as the single-stranded DNA binding protein observed by Sigal et al. , (1972) on the basis of its molecular weight in SDS gels and its failure to cause hyperchromicity of T4 DNA (Flintoff and Paetkau, 1974). It does not affect the transcription of T4 DNA (Flintoff and Paetkau, 1974) as does a protein of similar low molecular weight (Cukier-Kahn et al. , 1972).

The only known function of S factor is its blocking of the production of clc sequences during in vitro copying of DNA. It has been suggested that clc DNA may arise if the polymerase switches strands (Schildkraut et al. ,1964) or doubles back to copy the newly made strand (Harwood and Wells, 1970). It has been suggested that S factor acts by

binding to the displaced strand to prevent switching or doubling back (Flintoff and Paetkau, 1974). These mechanisms are shown in Figure 22.

It might also be postulated that S factor is the "knife" of Guild's (1968) "knife and fork" model of replication. Such a mechanism for S factor cannot be distinguished at present from the one shown in Figure 22a. A nucleolytic action at the growing fork would likely require a highly specific combination of template, primer, and polymerase existing only at the replicative site.

Natural DNAs have been used as substrates for S factor. The copying of E. coli DNA in the presence of S factor results in a decrease in the percentage of clc structures (Flintoff and Paetkau, 1974). PM2 DNA (RFII) provides a template in which strand switching plays a major role in the net-fold synthesis in vitro (Masamune and Richardson, 1971). S factor reduced but did not completely prevent clc DNA production. A consideration of Masamune and Richardson's (1971) model of in vitro copying of PM2 DNA may help to explain this (Figure 23). The equilibration between the various structures permits net-fold copying of the template. The equilibrium between Ia and Ib would be expected in the early stages when the displaced 5'-end is short. Both Ia and Ib could give rise to clc structures. In Ib a short region of intrastrand complementarity at the 3'-end may permit hydrogen bonding as was seen in Figure 4. If S factor does

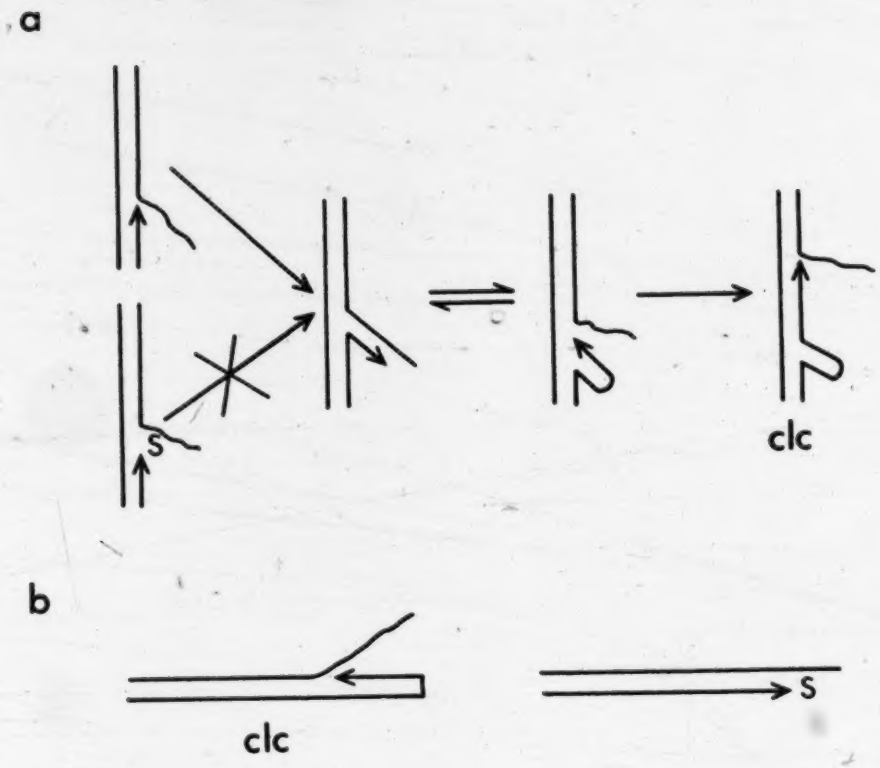


FIGURE 22. Models for clc DNA Production and the Action of S Factor. DNA polymerase may (a) switch strands or (b) double back.

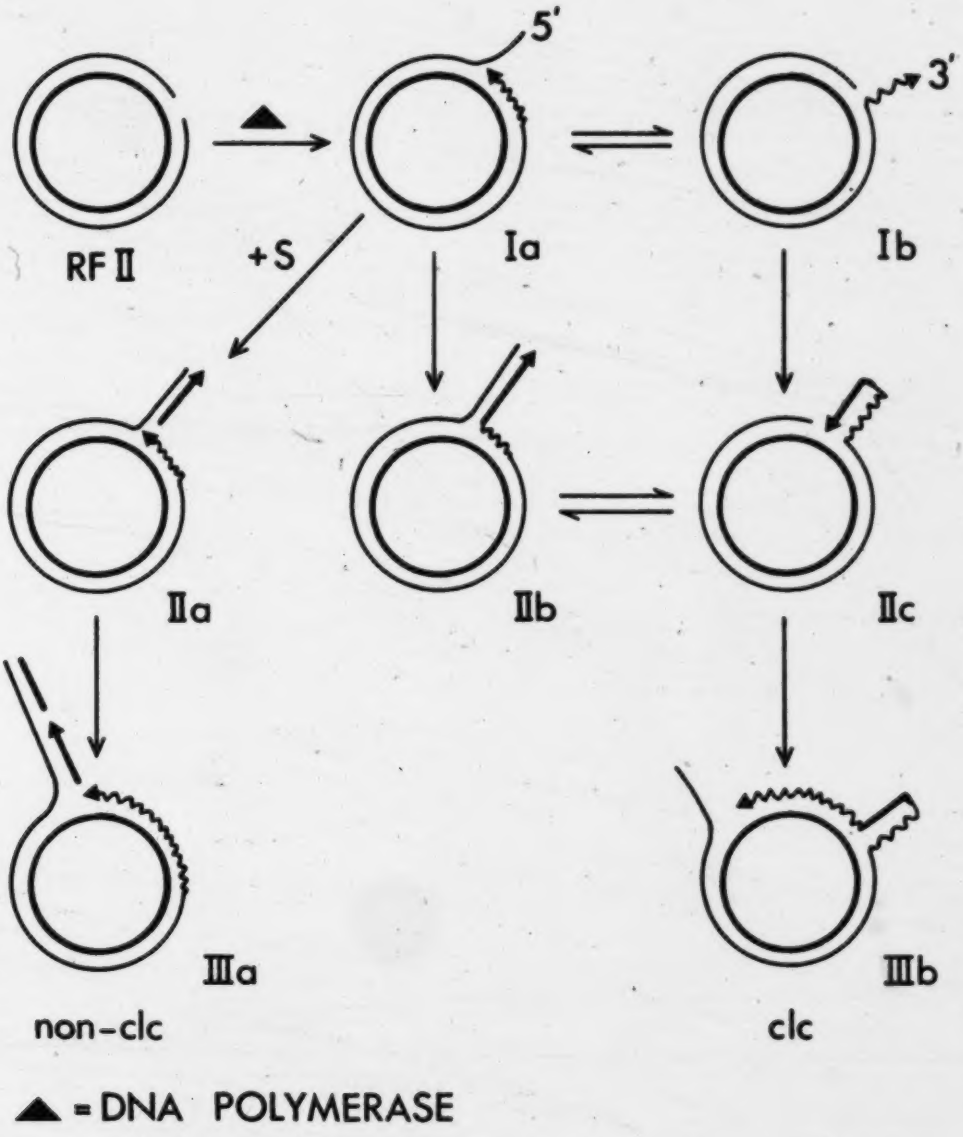


FIGURE 23. Clc DNA Production during PM2 DNA Synthesis in vitro .

not recognize structures such as Ib then clc DNA may be produced through an alternate means. In vivo the equilibrium between Ia and Ib would be blocked by initiation on the displaced 5'-strand.

The above model for S factor with circular DNAs could be tested using circular poly[d(T-G)•d(C-A)]. The equilibrium between structures Ia and Ib would still occur, however, lack of any intrastrand complementarity would prevent IIc from arising by copying. Clc structures should arise only via IIb. If S factor blocks IIb production, then IIa will be the product. The nature of the clc structures could be tested not only by fluorescence but, as with linear polymers, by alkaline CsCl density gradient centrifugation if the complementary sequences were differentially labelled. It is possible to prepare circular poly[d(T-G)•d(C-A)] using linear polymers and polynucleotide ligase (Paetkau and Khorana, 1971). The main problem is one of yield.

As stated in Chapter I, the scarcity of evidence for clc structures from in vivo replication is not suprising in view of their potential lethality. However, it may be possible to find conditional lethal mutants for the S factor function. If such a mutant were available, the effects on E. coli DNA or bacteriophage DNAs might prove interesting. It has been suggested (Flintoff, 1973) that one of the uncharacterized ts dna loci, for instance the dna C(D) locus whose product has a molecular weight of 25,000, may be

responsible for S factor. The properties of these mutants, particularly dna D, are under investigation to see if they tend to accumulate clc sequences *in vivo* under the non-permissive conditions.

It has also been suggested (Flintoff, 1973) that *E. coli* cells made permeable to antibodies with toluene and Triton X-100 (Moses, 1972) might be used to study the effects of inactivation of S by a specific antibody. Application of such a system to an *E. coli* strain carrying the polymerase I dependent circular plasmid colicin E1 may result in structures resembling those seen for PM2 DNA copied *in vitro* particularly since this colicin uses polymerase I for its DNA replication.

Bacteriophage or plasmid DNAs are especially useful because their small size makes them amenable to study in an undamaged form by electron microscopy.

CHAPTER V

UNUSUAL TEMPLATE ACTIVITIES IN EXTRACTS OF *E. coli*

I Introduction

Purified DNA polymerase I from *E. coli* synthesizes the copolymer, poly[d(A-T)•d(A-T)] and the homopolymer, poly[d(G)•d(C)] in apparently *de novo* reactions (Schachman *et al.*, 1960; Kornberg *et al.*, 1964; Burd and Wells, 1970; Radding *et al.*, 1962). The characteristics of such syntheses are the absence of an exogenous template, a long lag period varying from 2 to 8 hours, and the rapid synthesis of polymers containing only either dAMP and TMP in equal amounts, or dGMP and dCMP respectively. These reactions could be the result of either *de novo* synthesis or copying of undetected small oligodeoxynucleotides associated with the polymerase. The kinetics of synthesis of each polymer may be nearly exponential at early times and are typical of autocatalytic reactions beginning with very small templates (Radding and Kornberg, 1962).

The presence of an apparent template activity for poly[d(A-T)•d(A-T)] associated with S factor preparations has recently been reported (Flintoff and Paetkau, 1974). In addition, it has been observed that there is an apparent

template for polypyrimidine•polypurine DNA in the crude fraction, DIII (Morgan et al., 1974).

The existence of such templates provides one possible source for the apparently de novo reactions. The partial characterization of the template in DIII fractions and the complete characterization of the template associated with S factor are the topics of this chapter.

II Results: Template Activity in DIII

Unless otherwise stated the conditions for all synthetic reactions were those described for defined DNAs in Chapter II, with all four dNTPs present in equal proportions and no tRNA added. DIII is a by-product of a preparation of RNA polymerase by the method of Chamberlin and Berg (1962) (Chapter II).

A. Nucleic Acid Content of DIII

DIII had an absorbance of 10 A260 and an A260/A280 ratio of 1.0 before it was subjected to any treatment. Incubation with 0.1% SDS for 10 minutes at 50° followed by centrifugation reduced the absorbance to 3.0 A260 and increased the A260/A280 ratio to 1.4. Comparison to a standard sample of DNA in the fluorescence assay suggested a double-stranded DNA content of 0.03-0.07 A260.

B. Preparation of Oligonucleotide Fragments from DIII

High molecular weight material was removed from DIII preparations by chromatography of 1 ml samples of DIII on 15M Agarose columns (1x30 cm) equilibrated with 5 mM Tris-Cl (pH 8) - 0.1 mM EDTA. A typical elution profile is shown in Figure 24. Fraction 19, with an A260/A280 ratio of 1.4, was used as a source of template activity. This material was found to contain oligodeoxynucleotide fragments. The proportion of material in the included and excluded regions varied with different preparations of DIII.

C. Amplification of the Oligodeoxynucleotide Fragments by Copying

Fraction 19 was added to a standard synthesis mixture to a final concentration of 0.03 A260. The product was labelled with [^{3}H]TTP. Synthesis was monitored by the fluorescence assay. The results for copying fraction 19 as well as another sample of similarly prepared material are shown in Figure 25. The fraction 19 template showed a 4 hour lag period followed by rapid synthesis. The other template showed a lag period of less than one hour. The product of the fraction 19 template will be referred to as Polymer A; the product of the other template as Polymer B. The synthesis was stopped and the products isolated after 6 hours for Polymer A and 5 hours for Polymer B. A third polymer, Polymer C, was produced from a third DIII

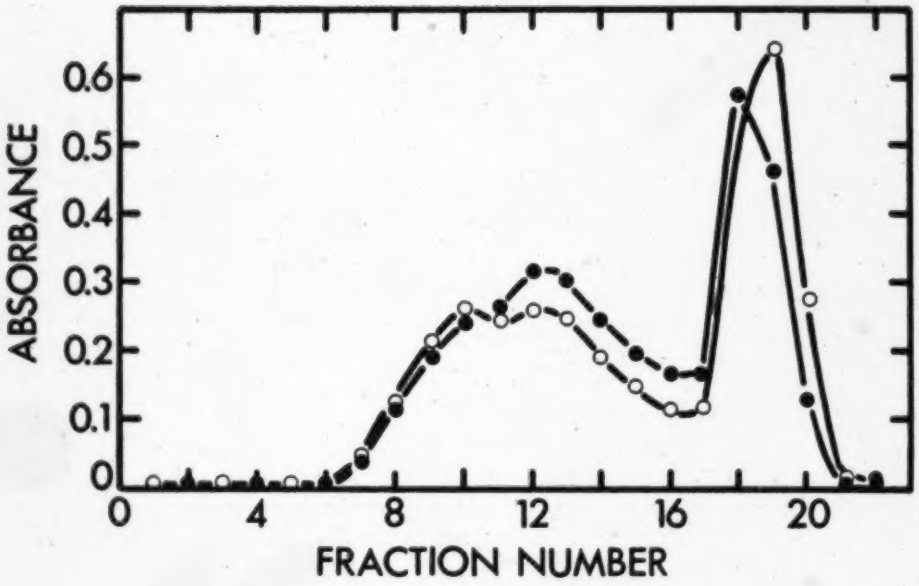


FIGURE 24. Agarose Chromatography (15M) of DIII. DIII was chromatographed on a 15M Agarose column (1x30 cm) equilibrated with 5 mM Tris-Cl (pH 8) - 0.1 mM EDTA. The column was run at room temperature by gravity with a flow rate of 7.6 ml/hour. Fractions were 1.9 ml. Open circles, A260; closed circles, A280.

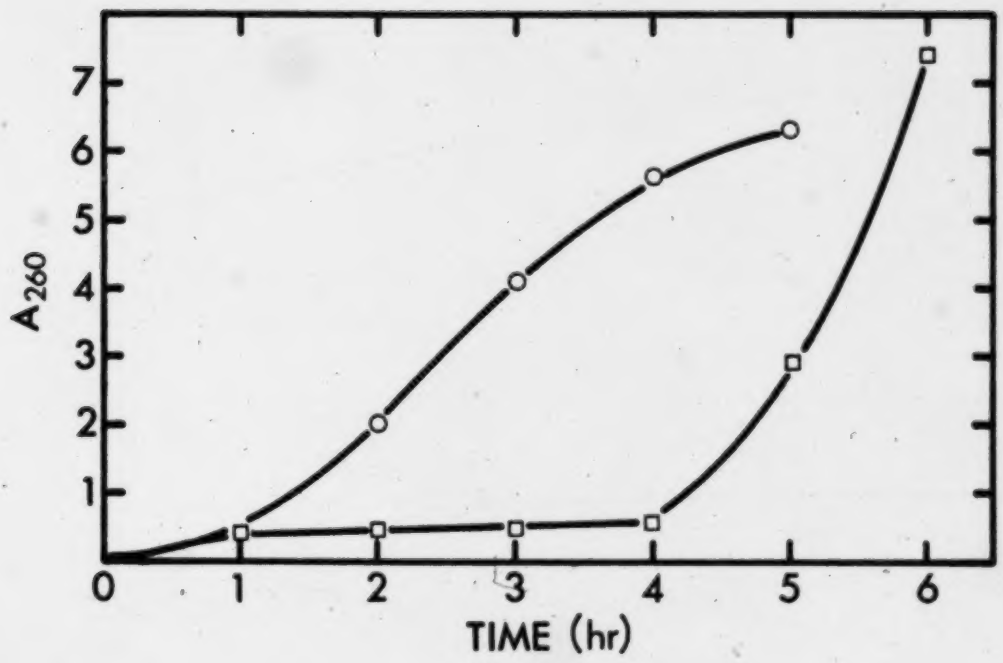


FIGURE 25. Copying of Oligodeoxynucleotide Fragments from DIII. Oligodeoxynucleotide fragments were added to a synthesis mixture containing equal proportions of all 4 dNTPs. The synthesis was monitored by comparison to a standard DNA in the fluorescence assay. The products were isolated by 15M Agarose chromatography and are designated Polymer A (fraction 19 template, squares) and Polymer B (circles). The initial concentration of template source was 0.03 for Polymer A and 0.05 A260 for Polymer B.

preparation. Its kinetics of synthesis were similar to those of Polymer A. Other amplification reactions showed considerable variation in the synthesis kinetics and ratios of [^{14}C]dCMP/[^{3}H]TMP incorporated.

D. Properties of the Isolated Products of the Amplification Reaction

(i) Molecular weight

Polymer A had a single-stranded molecular weight of 110,000 and a double-stranded molecular weight of 676,000 determined by alkaline and neutral sedimentation velocities respectively (Chapter II). This suggested a high molecular weight DNA product having several single-stranded nicks.

(ii) Clc DNA content

The clc DNA content of each isolated polymer was determined by fluorescence with the results: Polymer A, 41%; Polymer B, 30%; Polymer C, 29%.

(iii) Buoyant density

The buoyant density of Polymer A was determined by analytical neutral CsCl density gradient centrifugation (Chapter II). The polymer, in low salt, was heated in boiling water then quickly cooled before centrifugation. The buoyant density, determined by the isoconcentration method, was 1.810 g/cm^{3}. This is similar to the density of

poly[dG•dC], 1.794 g/cm³ (Erikson and Szybalski, 1964).

(iv) TMP content

Polymer A was labelled with [³H]TTP during its synthesis but the isolated product contained very little label. It was estimated that less than 5% of the residues were TMP.

(v) Template for poly[r(G)] synthesis and poly[r(C)] synthesis

All three polymers were tested for transcription by RNA polymerase to poly[r(G)] and poly[r(C)] (Chapter II). The results are shown in Figure 26. Polymers A and B had similar kinetics of poly[r(G)] synthesis, that is a rapid initial rate followed by a slower rate. Both polymers also showed poly[r(C)] synthesis. Polymer C was also transcribed to poly[r(G)] but the kinetics showed a slow initial rate follwed by a rapid rate. Polymer C gave very little poly[r(C)] transcription. There was essentially no poly[r(A)] or poly[r(U)] transcription for any of the three polymers. The templates used to produce Polymers A, B, and C were not themselves detectably transcribed.

The kinetics of transcription to poly[r(G)] for Polymers A and B were typical of a polymer having poly[d(C)] tracts (Paetkau et al., 1972). Extensive transcription to

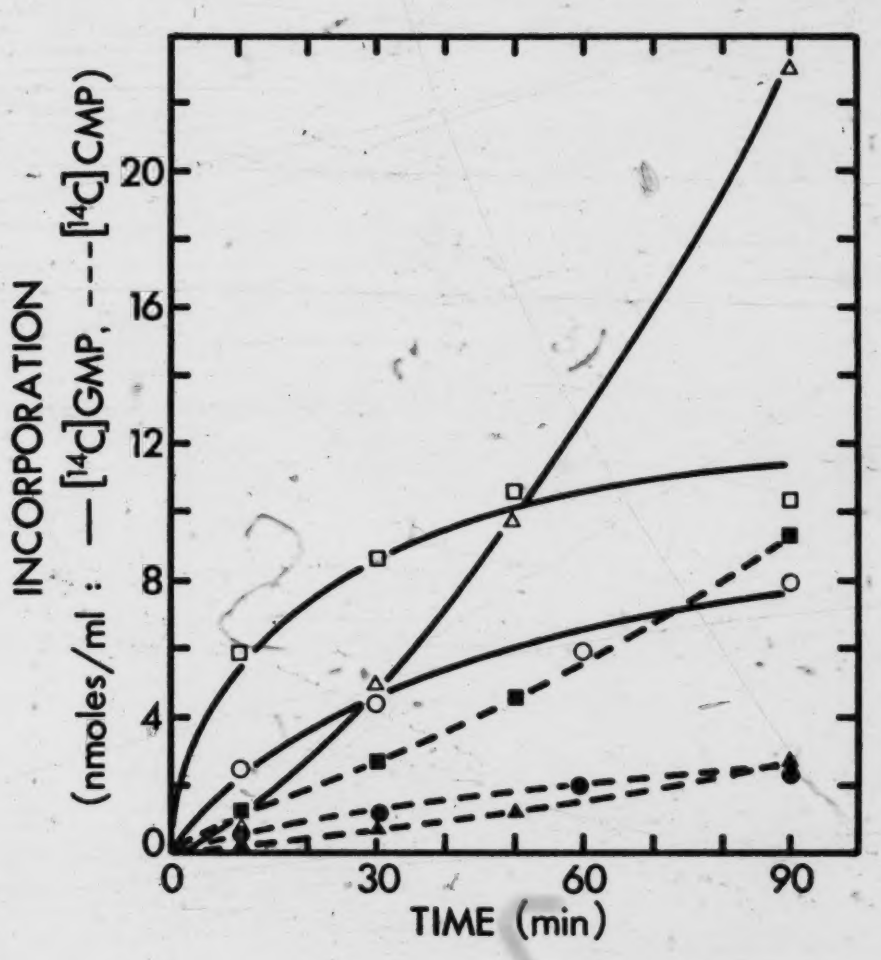


Figure 26. Transcription of Polymers A, B, and C to Poly[r(G)] and Poly[r(C)]. Polymers A, B, and C were transcribed by RNA polymerase with [^{14}C] CTP or [^{14}C]GTP as the only nucleotides present (Chapter II). Synthesis was monitored by incorporation of label into TCA-insoluble material. Solid lines, poly[r(G)] synthesis; broken lines, poly[r(C)] synthesis. Circles, Polymer A; squares, Polymer B; triangles, Polymer C.

poly[r(C)] suggested poly[d(G)] tracts. The kinetics of poly[r(G)] synthesis for Polymer C are those to be expected for a template containing T and dC residues in one strand. Polymers such as poly[d(T-C)•d(G-A)] and poly[d(T-T-C)•d(G-A-A)] are transcribed in this way (Paetkau et al., 1972).

III Results: Template Activity Associated with S Factor

S factor fractions were prepared as described in Chapter IV. Unless otherwise stated, all synthetic reactions were performed as described in Chapter II for poly[d(A-T)•d(A-T)] with tRNA present where indicated.

A. DNA Content of S Factor Fraction 6A

The presence of DNA in Fraction 6 or 6A was not detectable by measuring A260. By using the ethidium bromide fluorescence assay it was found that Fraction 6A contained approximately 0.002 A260 DNA (0.38 ng DNA/ug protein).

B. DNA Polymerase Content of Fraction 6

Various concentrations of Fraction 6 were tested in the usual synthetic reaction without added DNA polymerase. Fraction 6 contained no DNA polymerizing activity, either in the absence of added template or in the presence of an authentic poly[d(A-T)•d(A-T)] template.

C. Template Activity in S Factor Fractions

(i) Co-purification with S factor

The profile of the chromatography of Fraction 5 on G-75 Sephadex is shown in Figure 15 (Chapter IV). DNA polymerase-dependent template activity co-chromatographed with S factor in a partially included position. The division of this material into Fractions 6 and 6A was discussed in Chapter IV.

(ii) Template activity for apparent poly[d(A-T)•d(A-T)] synthesis

Polymers containing only dAMP and TMP were synthesized with DNA polymerase using either Fraction 6 or authentic poly[d(A-T)•d(A-T)] as a template. The results are shown in Figure 27. With authentic poly[d(A-T)•d(A-T)] template the lag period was less than one hour. Adding a high level of Fraction 6 resulted in a short lag period of 1 to 2 hours followed by rapid synthesis and subsequent degradation of the product. The lower concentration of Fraction 6 gave a slower rate of synthesis reaching a greater extent before degradation ensued. The stimulation of synthesis and the rapid degradation of the product was likely due to the nuclease activity of Fraction 6 of which about half was tRNA inhibitable endonuclease I (Table VII, Chapter IV). The addition of tRNA at 0.5 A260 to the synthetic reaction resulted in more extensive synthesis at the higher Fraction

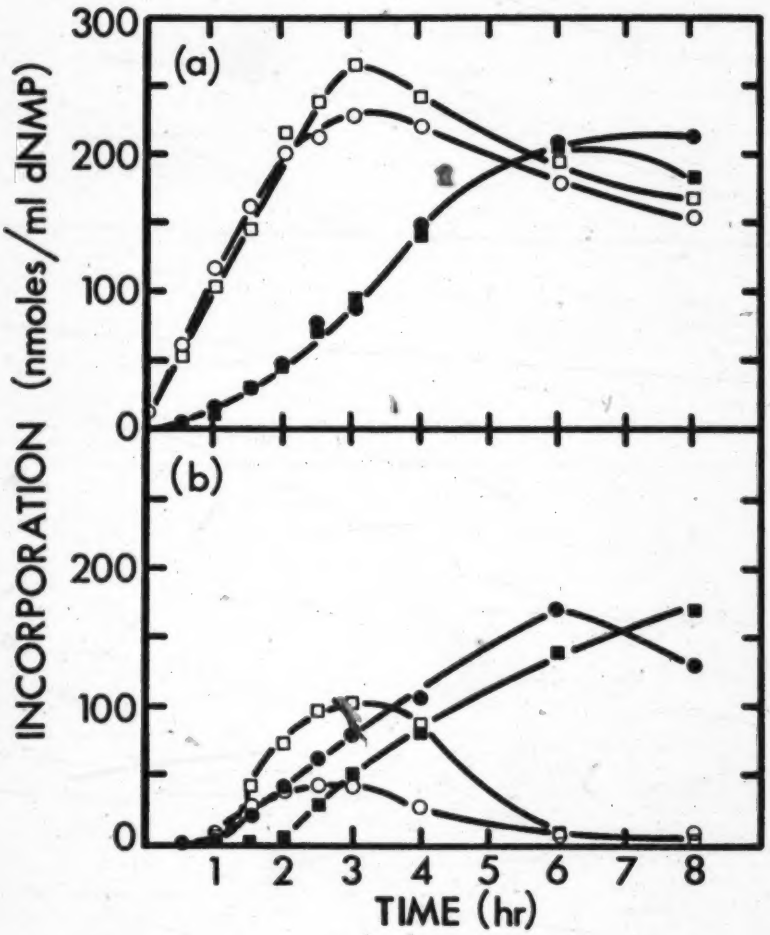


FIGURE 27. Copying of Poly[d(A-T)•d(A-T)] and Templates in S Factor. Synthesis was followed by incorporation of [^{3}H]TTP into TCA-insoluble material. Templates were provided by either (a) authentic poly[d(A-T)•d(A-T)]: open symbols, 0.2 A260 and closed symbols, 0.01 A260; or (b) S factor Fraction 6: open symbols, 11.5 ug/ml and closed symbols, 0.58 ug/ml. Circles, no tRNA; squares, 0.5 A260 tRNA.

6 concentration and a longer lag period at the lower Fraction 6 concentration. It had little effect on the copying of the authentic poly[d(A-T)•d(A-T)].

A comparison was made between the kinetics of copying the template in Fraction 6 and authentic poly[d(A-T)•d(A-T)], and the apparently de novo synthesis which occurs with no added templates. The results are seen in Figure 28. Synthesis did eventually occur in the absence of exogenous template, but addition of Fraction 6 reduced the lag period from 16 hours to 1 to 2 hours. In all subsequent experiments reported here there was no apparently template independent synthesis during the time course under observation.

D. Characterization of the DNA Polymerase Product of the S Factor Template

(i) Nucleotide composition of the product

The template present with S factor was copied with DNA polymerase in the presence of various combinations of dNTPs. The results are shown in Table XI. The only dNTPs which appeared to be required or incorporated were dATP and TTP. Synthesis did occur when all four dNTPs were present but no incorporation of [^{14}C]dCTP was detectable.

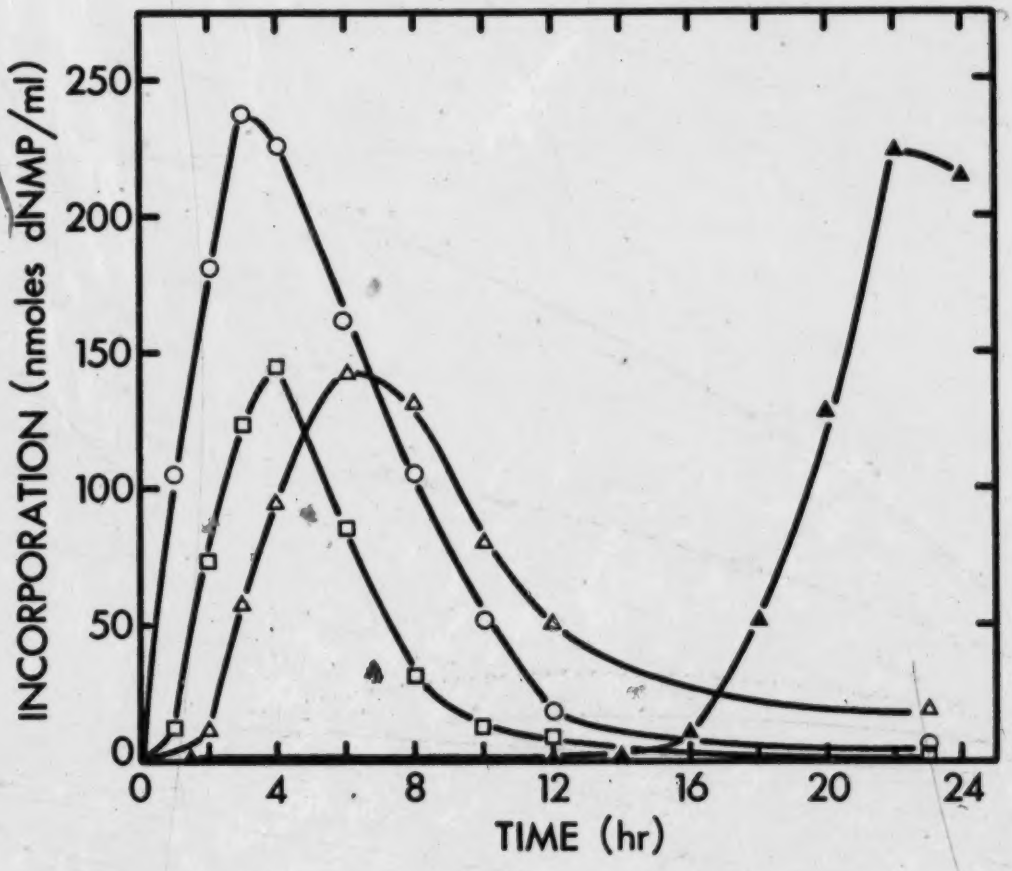


FIGURE 28. Comparison of Synthesis Using Exogenous Templates and an Apparently de novo Reaction. Synthesis was monitored by the incorporation of [^{3}H]TTP in the standard reaction with tRNA present in all cases. Templates were added as follows: circles, 0.1 A260 poly[d(A-T)•d(A-T)]; squares, 2.9 ug/ml Fraction 6; open triangles, 0.58 ug/ml Fraction 6; closed triangles, none.

TABLE XI

Nucleotide Composition of Product

Conditions[1]	dNMP Incorporation (nmoles/ml)[2] [^{3}H]TMP	[^{14}C]dCMP	DNA[2,3] Synthesis (nmoles/ml)	%[2,3] clc
[^{3}H]TTP, dATP	0	-	0[4]	-
0.2 A260 poly[d(A-T)•d(A-T)] +[^{3}H]TTP, dATP	97	-	-	94
Fraction 6 (20 ug/ml) +[^{3}H]TTP, dATP	33	-	66	98
+[^{3}H]TTP, [^{14}C]dCTP, dATP, dGTP	29	0	60	100
+[^{3}H]TTP	0	-	0	-
+[^{3}H]TTP, dCTP	0	-	0	-
+[^{3}H]TTP, dGTP	0	-	0	-
+[^{14}C]dCTP, TTP, dGTP, dATP	-	0	60	94

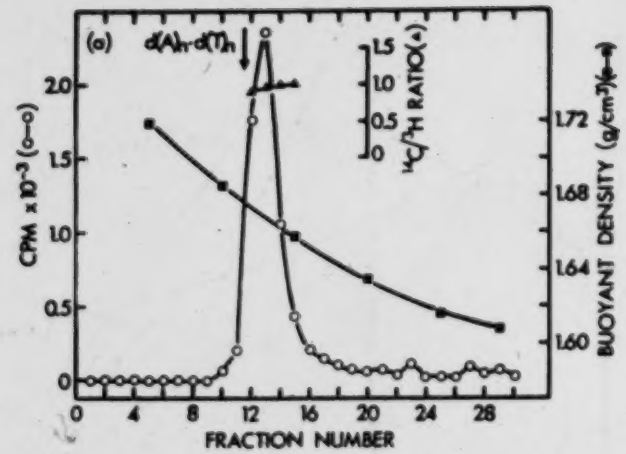
[1] All conditions included DNA polymerase at the usual concentrations. All dNTPs were at 0.2 mM.
[2] All values were measured at 2 hours.
[3] Values determined by fluorescence assay.
[4] No synthesis occurred during 6 hours.

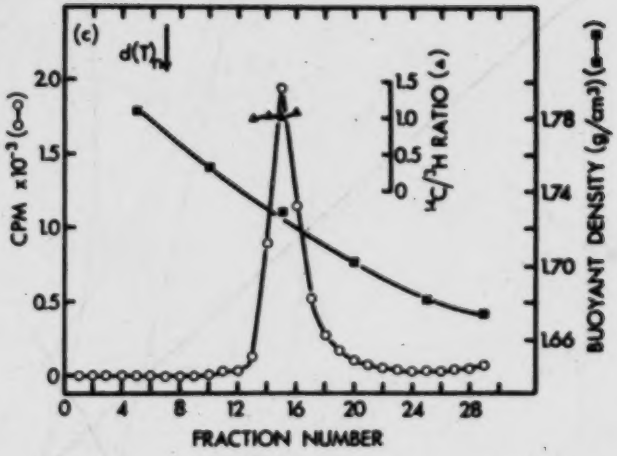
(ii) Physical characteristics of the product

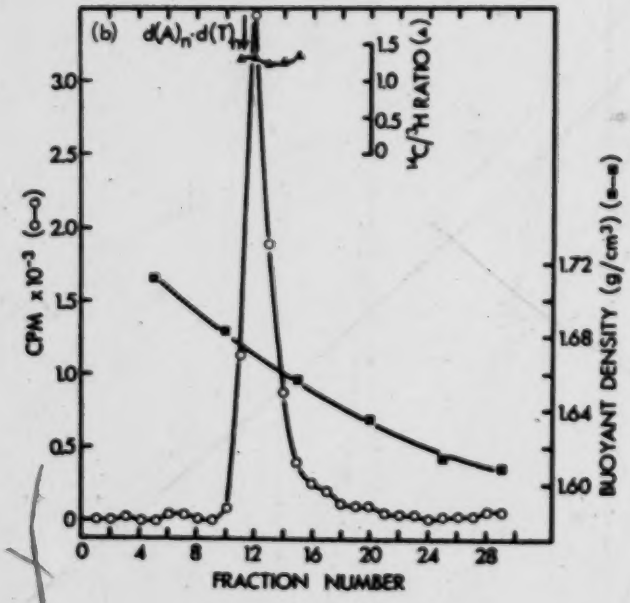
The product was compared to authentic poly[d(A-T)•d(A-T)] in terms of its physical characteristics. Four methods were used:

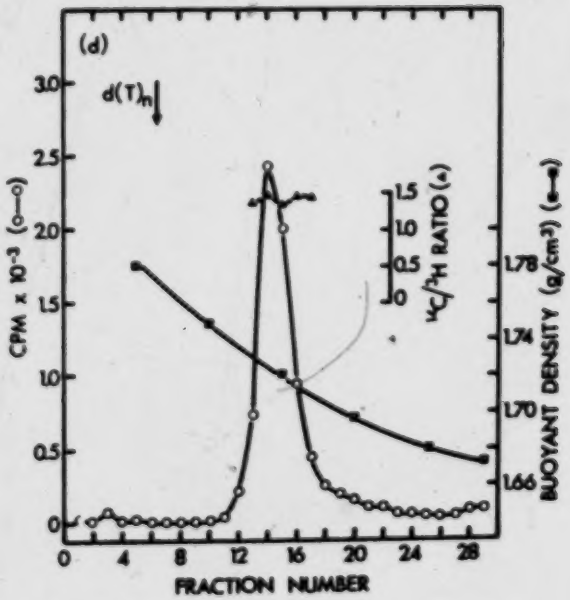
a). Buoyant density. The buoyant densities in CsCl were compared for the products copied from the Fraction 5 template and from authentic poly[d(A-T)•d(A-T)] under the usual conditions with 0.5 A260 present. The products were labelled with [^{14}C]TTP and [^{3}H]dATP. The extent of synthesis was determined by the fluorescence method. The reaction was stopped after 3.5 hours for the authentic poly[d(A-T)•d(A-T)] template and 6.5 hours for the Fraction 5 template. The resulting material was added directly to neutral and alkaline CsCl solutions and centrifuged as indicated in Figure 29. There was no separation of ^{3}H- and ^{14}C-labels either in alkaline or neutral gradients. The buoyant densities of the product made from the Fraction 5 template corresponded to those seen for the authentic poly[d(A-T)•d(A-T)] template in both gradients. The sharpness of the bands seen in the gradients suggested that the material was of high molecular weight. Another experiment in which tRNA was absent resulted in a broad band of material from the Fraction 5 template. This band centered at the density of the authentic poly[d(A-T)•d(A-T)] in both alkaline and neutral gradients.

FIGURE 29. CsCl Density Gradient Centrifugation of Poly[d(A-T)•d(A-T)] and the Product of the Fraction 5 Template. The polymers were synthesized using 4 ug/ml Fraction 5 or 0.01 A260 authentic poly[d(A-T)•d(A-T)] as templates. The neutral CsCl solutions contained 20 mM Tris-Cl (pH 8) - 1 mM EDTA and CsCl to a density of 1.658 g/cm^3. The alkaline solutions contained 50 mM NaOH - 1 mM EDTA and CsCl to a density of 1.725 g/cm^3. The solutions were centrifuged in a fixed angle Ti 50 rotor at 38,000 rpm for 88 hours at 20°. Fractions of 0.2 ml were pumped from the bottoms of the tubes. Aliquots of 50 ul were removed from each fraction to determine TCA-insoluble cpm. Densities were determined refractometrically. Polymer copied from the Fraction 5 template: (a) neutral, (c) alkaline gradients; polymer copied from authentic poly[d(A-T)•d(A-T)]: (b) neutral, (d) alkaline gradients. The arrows indicate the buoyant densities expected for poly[d(A)•(T)] (Wells and Blair, 1967). Circles, total acid insoluble cpm; squares, buoyant density; triangles, ratio of [^{14}C]TMP/[^{3}H]dAMP.


(a)
d(A)n-d(T)n
CPM x10⁻³ (o—o)
BUOYANT DENSITY (g/cm³)(•—•)
¹⁴C/³H RATIO(▲)
FRACTION NUMBER


(c)
d(T)n
CPM x10⁻³ (o—o)
BUOYANT DENSITY (g/cm³)(•—•)
¹⁴C/³H RATIO (▲)
FRACTION NUMBER


(b)
d(A)n-d(T)n
CPM x10⁻³ (o—o)
BUOYANT DENSITY (g/cm³) (•—•)
¹⁴C/³H RATIO (▲)
FRACTION NUMBER


(d)
d(T)n
CPM x 10⁻³ (o—o)
BUOYANT DENSITY (g/cm³) (•—•)
¹⁴C/³H RATIO (▲)
FRACTION NUMBER

b). Temperature-absorbance profiles. The Tm's were compared for the products of the Fraction 6 template and the authentic poly[d(A-T)•d(A-T)] template. The two products were prepared in the usual way with tRNA present and were isolated after 4.5 hour of synthesis. For one polymer the template was 0.01 A260 of authentic poly[d(A-T)•d(A-T)]; for the other 0.6 ug/ml of Fraction 6 protein. The Tm's were measured in 5 mM potassium phosphate (pH 6.8) - 0.1 M EDTA - 0.1 M NaCl. The polymers had identical melting curves each with a Tm of 63° and a total of 50% hyperchromicity.

c). Molecular weight. The molecular weight of a sample of the Fraction 6 product prepared above was determined by alkaline and neutral sedimentation velocity (Chapter II). The product had single- and double-stranded molecular weights of 98,000 and 138,000 respectively. This indicated a double-stranded product probably having "hairpin" structures.

d). Clc DNA content. The percentage of clc DNA was 90-100% for products of the Fraction 6 template and authentic poly[d(A-T)•d(A-T)]. This is to be expected for a completely self-complementary polynucleotide like poly[d(A-T)•d(A-T)] (Morgan and Paetkau, 1972)

These results taken together suggested that the product of the template in S factor fractions was a high molecular weight product which was physically indistinguishable from

authentic poly[d(A-T)•d(A-T)].

(iii) Nearest neighbour analysis of the product

Samples of the same polymers used for the Tm determinations were subjected to formic acid-diphenylamine depurination and degradation, followed by paper electrophoresis (Chapter II). Half of each degraded sample was treated with bacterial alkaline phophatase. The results, shown in Figure 30 were the same for both polymers. Without alkaline phosphatase treatment, essentially all the labelled material migrated to the position expected for 3',5'-thymidine diphosphate. Partial digestion with alkaline phosphatase converted all the labelled material to a mixture of thymidine and thymidine monophosphate. If the product of the Fraction 6 template had contained random sequences of T and dA, the degradation would have yielded fragments of oligo[d(T)] bearing 5'- and 3'-phosphoryl groups. Such fragments would not migrate as thymidine diphosphates before alkaline phosphatase treatment nor as thymidine and thymidine monophosphate after partial phosphatase treatment. These results confirmed that the product of the Fraction 6 template was a strictly alternating copolymer of dA and T.

E. Characterization of the Template in S Factor Fractions

(i) Stability in alkali and pronase

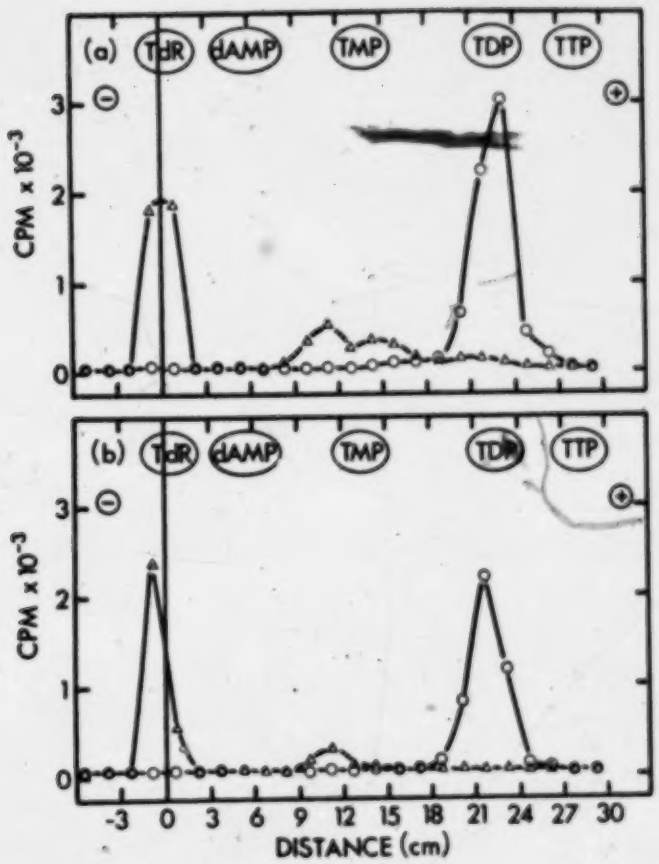


FIGURE 30. Electrophoresis of Depurinated and Degraded Poly[d(A-T)•d(A-T)] and the Product of the Fraction 6 Template. Depurination, degradation, alkaline phosphatase treatment, and electrophoresis (Chapter II) were performed on (a) 0.17 A260 units of authentic poly[d(A-T)•d(A-T)] and (b) 0.10 A260 units of the product of the Fraction 6 template. Samples were subjected to paper electrophoresis with 0.1 M sodium citrate (pH 4.15) - 1 mM EDTA buffer at 1500 V for 1.5 hours at 22°. The labelled components were located as described in Chapter II. The migration positions of unlabelled marker compounds are indicated. Circles, no alkaline phosphatase treatment; triangles, partial alkaline phosphatase digestion.

A sample containing 40-50 ug/ml Fraction 5 protein in buffer H (20 mM Tris-Cl (pH 8) 1 mM EDTA) was made 0.3 N in KOH and incubated at 37° for 19 hours. Aliquots were removed at various times during the incubation, neutralized with acetic acid, then boiled for 2 minutes. The aliquots were added to the usual synthesis mixtures. The extent of synthesis was determined by incorporation of [^{3}H]TTP. The results are shown in Figure 31. Under the conditions of alkali treatment used here, RNA should have been hydrolyzed and protein denatured. The inactivation of proteins was evident in the change in the kinetics of synthesis - the lag period increased, presumably because of the destruction of endogenous nucleases. However, the template for poly[d(A-T)•d(A-T)] synthesis persisted. In a separate experiment it was shown that the synthesized material was 95-100% clc DNA.

A sample containing 40-50 ug/ml Fraction 5 protein and 1 mg/ml pronase was incubated at 40° for 4 hours. The sample was then boiled for 3 minutes. Template activity was tested in the usual way and monitored by incorporation of [^{3}H]TTP. The results are shown in Figure 32. Template activity was still present after the pronase treatment. The destruction of nucleases was again evident from the extended lag phase, the greater extent of synthesis, and the absence of rapid degradation of the product.

The resistance of the template activity to alkaline hydrolysis and pronase digestion suggested that it resides

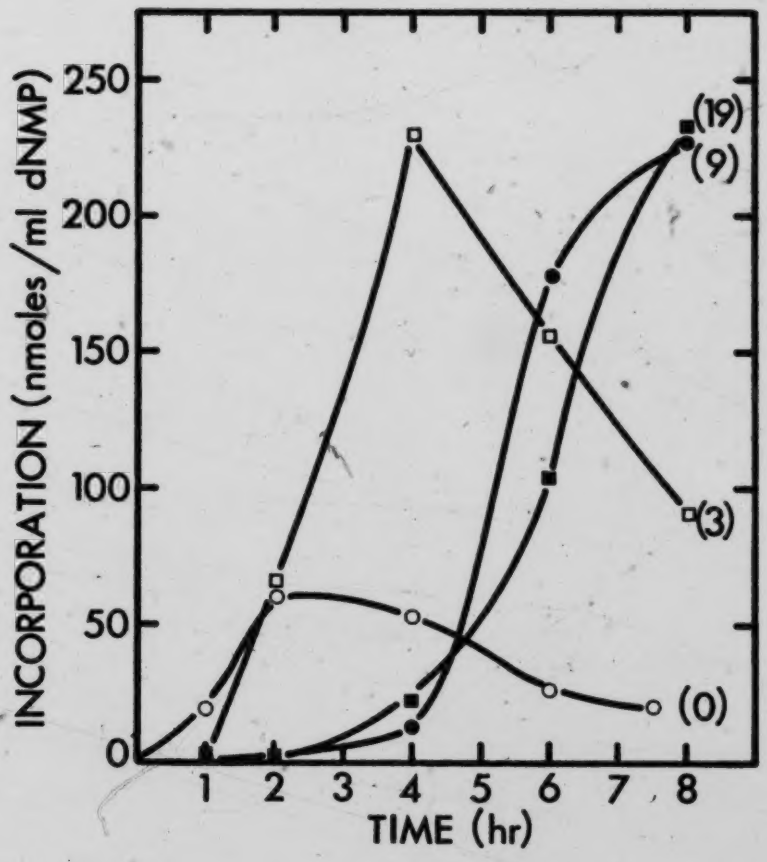


FIGURE 31. Effect of Alkaline Hydrolysis on the Fraction 5 Template. Alkaline hydrolysis was performed as indicated in the text. Neutralized and boiled aliquots were diluted 1/10 into a synthesis mixture containing [^{3}H]TTP. The numbers in parentheses represent the hours of exposure to alkali before the aliquot was removed for synthesis testing.

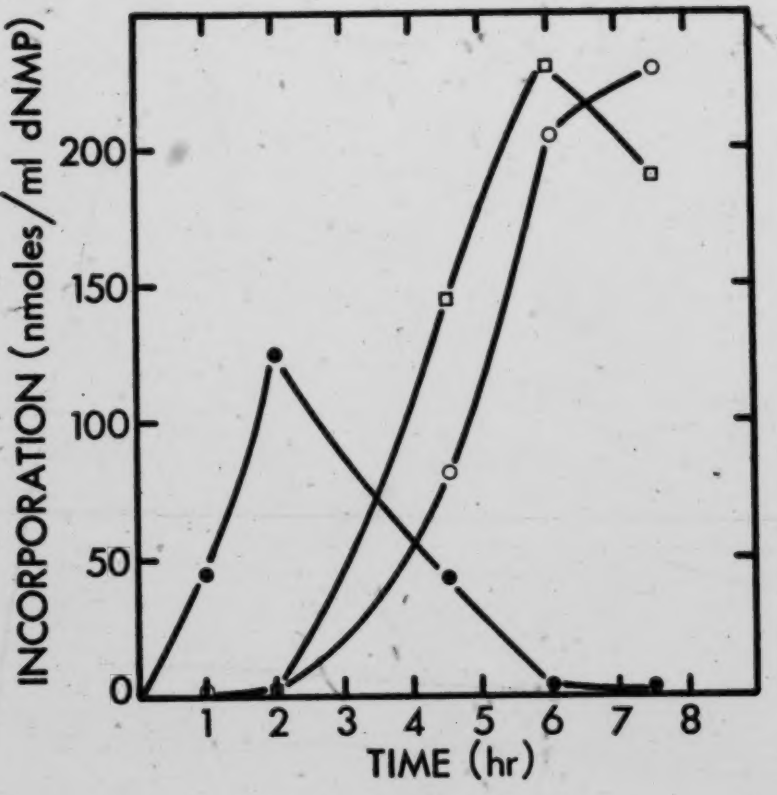


FIGURE 32. Effect of Pronase on the Fraction 5 Template. Fraction 5 was incubated with pronase and then tested for template activity in the usual way. Closed symbol, untreated Fraction 5 at 25 ug/ml. Open symbols, pronase-treated Fraction 5 diluted (1/10, circles; 1/50, squares) into the synthesis mixture.

in an oligodeoxynucleotide.

(ii) Effect of nucleases on the template

The persistence of the poly[d(A-T)•d(A-T)] template activity in S factor fractions after magnesium-dependent autolyses (Chapters II and IV) suggested a resistance to endogenous nucleases. To test this further, a sample containing 30-35 mg/ml Fraction 5 was subjected to the second autolysis but for a longer period of time. Samples taken at various times during the autolysis were boiled for 3 minutes then tested for template activity in the usual way. The results are shown in Table XII. Exogenous poly[d(A-T)•d(A-T)] at 0.15 A260 in a parallel sample was rendered more than 90% acid-soluble by 5 hours. However, the template in Fraction 5 was not significantly altered.

The effects on the template of various exogenous nucleases were tested:

a). Exonuclease III. A sample containing 1.6 mg/ml Fraction 6, 50-100 units/ml exonuclease III and 4 mM magnesium chloride was incubated at 30° for 3 hours. Aliquots taken at various times were boiled for 3 minutes and tested for template activity. The results are shown in Table XII. Exogenous [^{3}H-T]poly[d(A-T)•d(A-T)] at 0.25 A260 in a parallel sample was degraded more than 90% in 0.25 hours. The template activity of Fraction 6 was essentially unchanged.

TABLE XII

Nuclease Sensitivity of the Poly[d(A-T)•d(A-T)] Template

Nuclease	Template Source	Treatment Time (hr)	Synthesis[1]	Lag Time (hr)
Endogenous (autolysis)	Fraction 5 (1/10)[2]	0	50.0	2
		1.5	39.2	2
		3.0	45.3	2
		5.0	36.2	2
Exonuclease III	Fraction 6 (1/10)	0	33.5[4]	1
		0.75	28.3	2
		2.0	31.8	2
		3.5	31.0	2
DNase I	Fraction 6 (1/10)	3.0	40.2[4]	<1
DNase I + venom phosphodiesterase	Fraction 6 (1/10)	3.0	35.2[4]	<1
None[6]	Fraction 6 (1/10)	3.0	39.1[4]	<1
DNase I + venom phosphodiesterase (after alkali)	Fraction 6A (1/10)	3.0	0[5]	>8
None[6] (after alkali)	Fraction 6A (1/10)	3.0	40.3[5]	3-4

[1]Incorporation of dNMP (nmoles/ml).
[2]Dilution of treated sample into synthesis mixture.
[3]Incorporation at 6 hours.
[4]Incorporation at 4 hours.
[5]Incorporation at 8 hours.
[6]Samples were treated exactly as the parallel nuclease-treated sample except for the addition of nuclease.

b). DNase I and venom phosphodiesterase. Fraction 6 at 2 mg/ml of protein was incubated at 37° with 5 mM magnesium chloride and 5 ug/ml pancreatic DNase I or 5 ug/ml DNase I plus 50 ug/ml venom phosphodiesterase. In the latter case, an equivalent amount of fresh venom phosphodiesterase was added at 1.5 hours. After 3 hours, EDTA was added to 10 mM and the samples were boiled for 2 minutes before adding to the standard synthesis mixture. A parallel sample to which no nucleases had been added was also tested. The results are shown in Table XII. In a separate experiment it was determined that 0.25 A260 [^{3}H-T]poly[d(A-T)•d(A-T)] was degraded to 90% in 2 hours by the DNase I alone at the above concentration while the DNase I - venom phosphodiesterase combination resulted in more than 90% degradation in 0.25 hours. There was, however, little alteration in the template activity of Fraction 6.

The resistance of the template activity to nucleolytic attack suggested that it was protected, perhaps by binding to proteins such as S factor. Therefore alkali pretreatment was used to denature the protein before nuclease treatment. A sample of Fraction 6A containing 260 ug/ml of protein in buffer H was made 0.3 N in KOH and incubated for 17 hours at 37°. The sample was neutralized and adjusted to pH 8 with acetic acid. Nuclease treatment was then performed exactly as before with DNase I and venom phosphodiesterase. A

parallel alkali-treated sample had no nuclease added. The reactions were stopped by the addtion of EDTA to 10 mM and boiling for 2 minutes before testing for poly[d(A-T)•d(A-T)] synthesis. The results are shown in Table XII. The nuclease-treated material showed no incorporation during 8 hours of incubation. A similar result was obtained when nucleases were removed before the synthesis test by extraction with 0.5 volumes of chloroform:isoamyl alcohol (24:1). The denaturation of protein by the KOH treatment appeared to render the Fraction 6A template susceptible to nucleolytic attack.

Taken together these results indicate that the template is an oligodeoxynucleotide which is sensitive to nuclease action only after the removal of protecting proteins.

F. Association of the Poly[d(A-T)•d(A-T)] Template with S Factor

S factor activity and the template activity co-chromatograph on G-75 Sephadex and remain associated even in a urea-LiCl-CsCl density gradient (Figures 15, 20; Chapter IV). The template activity of Fraction 9 (0.28 ug protein/ml), consisting of at least 90% S factor (Chapter IV), was compared to that of Fraction 6 (2.9 ug protein/ml). Synthesis was monitored by the incorporation of [^{3}H]TTP. The incorporation after 8 hours was 225 nmoles total dNMP for Fraction 9 and 280 nmoles total dNMP for the Fraction 6

template. These results suggested that the template was strongly associated with S factor itself.

G. Poly[d(A-T)•d(A-T)] Template Activity in Natural DNA

The capacity of natural DNAs to serve as templates for poly[d(A-T)•d(A-T)] synthesis was tested in the usual way with tRNA present. Templates were provided by native and denatured E. coli, T7, and calf thymus DNAs at 0.1 A260. The templates were denatured by boiling for 2 minutes followed by rapid cooling in ice. In all cases there was no decrease in the lag period seen with the DNA polymerase alone, i.e., these DNAs had no template activity for poly[d(A-T)•d(A-T)] synthesis.

IV Discussion

The crude fraction, DIII has been found to contain apparent templates for the DNA polymerase-mediated synthesis of DNA polymers resembling poly[d(G)•d(C)] or polypyrimidine•polypurines. Evidence for this conclusion about their composition comes from the buoyant density of one such polymer in neutral CsCl but especially from the transcription to poly[r(G)] by RNA polymerase. This reaction is known to occur in the presence of DNAs having only dCMP or dCMP and TMP residues in one strand (Paetkau et al., 1972). The kinetics of the reaction reflect the frequency of TMP residues. The transcription to poly[r(C)] is indicative

of dGMP tracts.

The template in S factor preparation has been more completely characterized. The template itself is stable to alkali and pronase. It is sensitive to nucleolytic attack but only after deproteinization. The template therefore appears to be an oligodeoxynucleotide. It is very firmly bound to S factor and has probably been preserved through this association. The product copied from this template is indistinguishable from authentic poly[d(A-T)•d(A-T)] in its physical properties and nucleotide sequence. The sequence was confirmed by nearest neighbour analysis using a formic acid-diphenylamine degradation.

The apparently de novo synthesis of poly[d(A-T)•d(A-T)] or poly[d(G)•d(C)] has been assumed to require no template (Schachman et al., 1960; Radding et al., 1962; Kornberg et al., 1964; Burd and Wells, 1970). Models for the de novo production of high molecular weight polymers have usually involved, as a first step, the random or specific synthesis of oligomers which are favoured as templates (Kornberg et al., 1964; Burd and Wells, 1970). The high molecular weight polymer is then generated through copying the oligomers by the reiterative mechanism or the staggered mechanism (Figure 2, Chapter I). Burd and Wells (1970) observed that the M. luteus polymerase also performed a de novo synthesis producing a mixture of poly[d(A-T)•d(A-T)] and poly[d(A)•d(T)]. The proportion of each polymer in the

mixture depended on the precise reaction conditions.

The apparently de novo synthesis could be due to copying of DNA fragments associated with the polymerase. It is significant that highly purified E. coli DNA polymerase does not produce poly[d(G)•d(C)] de novo (Burd and Wells, 1970). Octanucleotides of oligo[d(A-T)] will serve as templates for poly[d(A-T)•d(A-T)] synthesis after a lag period (Kornberg et al., 1964). Small numbers of very short fragments could readily serve as templates but might escape detection unless amplified by copying. The templates in the DIII and S factor fractions may represent such fragments.

The origin of these templates is difficult to assess directly. A poly[d(A-T)•d(A-T)] template capacity of bulk E. coli DNA was undetectable, confirming the result of Kornberg et al. (1964). The value determined for the DNA content of Fraction 6A can be used to obtain a minimum estimate of 3 segments of 8 or more dAMP and TMP residues per E. coli cell assuming complete recovery. A similar estimate for DIII may be at least tenfold higher. These estimates are well within the range of the numbers that could arise by random assortment of the bases. A high affinity of certain DNA metabolizing proteins for the oligomers could result in the selection and preservation of particular sequences during the isolation of the proteins. The template for poly[d(A-T)•d(A-T)] may be associated with DNA polymerase as well as being bound and protected by S factor. The DIII fraction

comes from an RNA polymerase preparation. Considering the correlation between polypyrimidine•polypurine DNA segments and RNA transcription (Chapter I), it is possible that the DIII templates at one time were associated with RNA polymerase. The irreproducibility of the product composition may reflect the actual composition of the fragments or a random favouring of one sort of template by the DNA polymerase. The association of certain proteins with specific sequences in DNA may be an indication of preferred binding sites for the proteins.

Obviously many questions about these templates are left open. The template activity in DIII requires complete characterization with respect to the nature of the template(s) and the composition of its products. It is unusual that polypyimidine•polypurine DNA or poly[d(G)•d(C)] should have clc DNA content (Chapter III). Nearest neighbour analyses with [alpha-^{32}P]dNTPs may clarify this. It may be possible to analyze the DIII for oligopyrimidine DNA by fractionating for size and composition on DEAE-cellulose columns (Cerny et al., 1968).

The stability of the association between the poly[d(A-T)•d(A-T)] template and S factor raises the possibility, however unlikely, of a covalent linkage. One way to test this would be to heat the complex in Sarkosyl then centrifuge in a CsCl density gradient. The DNA would be detectable by its template activity and the protein by its

SDS gel electrophoresis pattern or its activity. If the bond is covalent, the DNA and protein would band together.

Binding of high molecular weight poly[d(A-T)•d(A-T)] by S factor was not demonstrable (Chapter IV). This may be largely a problem of the size of the poly[d(A-T)•d(A-T)]. Binding of oligo[d(A-T)] by S factor might be tested using labelled oligo[d(A-T)] of defined size. If bound the oligomer should co-chromatograph with S factor of G-75 or G-100 Sephadex and be protected from nucleases. The optimal size for binding may give an indication of the size of the template as isolated with S factor. The binding of oligo[d(A-T)] by other DNA metabolizing proteins might also be tested in this way.

CHAPTER VI

SUMMARY AND DISCUSSION

Clc DNA accumulates in the defined DNAs poly[d(T-G)•d(C-A)] and poly[d(T-T-G)•d(C-A-A)] synthesized *in vitro* in the absence of S factor activity. Clc structures are detectable by an ethidium bromide assay specific for double-stranded DNA and by alkaline CsCl equilibrium centrifugation. A non-clc poly[d(T-T-G)•d(C-A-A)] has been prepared from a clc polymer by copying the unlinked complementary strands after their physical separation from clc strands in an alkaline cesium sulfate density gradient.

The introduction of incorrect sequences into polymers prepared in the presence of crude S factor fractions or DIII is a problem especially with poly[d(T-T-G)•d(C-A-A)] since it is copied less efficiently than poly[d(T-G)•d(C-A)]. S factor fractions may result in poly[d(A-T)•d(A-T)] synthesis; DIII in polypyrimidine•polypurine DNA or poly[d(T,G)•d(C,A)] synthesis. Methods that have been used for the detection of anomalous sequences include transcription by RNA polymerase, density gradient centrifugation, Tm, and deoxynucleotide ratios. Incorrect sequences are best avoided by the use of highly purified

components in the synthesis mixture.

A protein, S factor, has been purified which reduces the production of clc sequences during the in vitro copying of the defined DNAs poly[d(T-G)•d(C-A)] and poly[d(T-T-G)•d(C-A-A)], and the natural DNA PM2 RFII. Clc DNA is thought to arise either by the polymerase switching strands to copy the displaced strand (Schildkraut et al., 1964) or its turning around to copy the newly made strand (Harwood and Wells, 1970). It has been suggested that S factor acts by binding to the displaced strand to prevent strand switching or reversal by the polymerase (Flintoff and Paetkau, 1974). These models are illustrated in Figure 23 (Chapter IV). In the case of the circular DNA, PM2 RFII, the reduction in clc DNA is neither immediate nor complete. A model has been proposed as a possible explanation of S factor's action on PM2 DNA (Figure 23, Chapter IV).

S factor does not affect repair synthesis of single-stranded DNA nor does it have a proteolytic effect on DNA polymerase. The S protein has a molecular weight of 9500-12,000 on SDS gel electrophoresis and an apparent molecular weight of 24,000-26,000 on G-75 Sephadex.

The purification of S factor is complicated by its association with a template for poly[d(A-T)•d(A-T)]. The template is not eliminated by mild denaturing conditions. It appears to be firmly bound to S factor. Binding of high

molecular weight poly[d(A-T)•d(A-T)] by S factor was not detectable.

Fractions used as sources of S factor contain templates for the synthesis of unusual DNAs. DIII contains template activities for polymers resembling polypyrimidine•polypurine DNA or poly[d(G)•d(C)]. S factor fractions contain a template for synthesis of a DNA which was identified as poly[d(A-T)•d(A-T)] by physical and chemical means. The template, stable to alkali and pronase, is an oligodeoxynucleotide and appears to be bound to and protected by S factor. It is sensitive to nucleolytic attack only after deproteinization.

Defined DNAs have been used extensively in this work. Their DNA-like properties and chemically defined sequences make them useful model substrates for DNA polymerase and other DNA metabolizing proteins. However, it is necessary to be aware of some of the problems associated with their polymerase-mediated synthesis - in particular, the accumulation of incorrect sequences sometimes to the almost complete exclusion of the proper sequences. Nearest neighbour analysis would be the least ambiguous single test for sequence. Combinations of several of the other techniques described here (Chapter III) are necessary to characterize the polymers.

The defined DNA, poly[d(T-G)•d(C-A)], has been

especially useful as a substrate for S factor. However, S factor's activity is not limited to defined DNAs. It is also effective with natural DNAs from E. coli (Flintoff and Paetkau, 1974) and PM2. There is little evidence that clc structures are produced in replicating DNA in vivo (Chapter I). The potential lethality of persistent clc DNA suggests that there is some specific mechanism either to remove clc structures or to prevent them from forming. The observation that, without S factor, the in vitro copying of natural DNAs from B. subtilis, T7, E. coli (Schildkraut et al., 1964; Coulter et al., 1974), and PM2 leads to rapidly renaturable DNA indicates an incomplete reconstitution of the in vivo replication mechanism. The connection between S factor's action in vitro and the absence of clc DNA in vivo is an obvious one but as yet there is no evidence that S factor serves this function in vivo. It is suggested in Chapter IV that cells permeable to antibodies might be useful for determining the in vivo function of S factor. This might also be tested in a concentrated bacterial lysate on cellophane membrane discs (Schaller et al., 1972).

The occurrence of template activities in DIII and S factor fractions has been of interest in light of their association with DNA metabolizing proteins and their possible role as templates for the apparently de novo reactions of DNA polymerase. The unusual sequences of these templates and their preservation suggests there has been a

selection for them perhaps through their association with specific proteins. There is evidence that some proteins recognize specific sequences in DNA. For example, dA-T rich DNA may be involved in binding of the lactose repressor and RNA polymerase (Lin and Riggs, 1970; Heyden et al. , 1972; LeTalaer and Jeanteur, 1971; Jones and Berg, 1966). Polypyrimidine tracts are correlated with the asymmetry of transcription (Summers and Szybalski, 1968; Champoux and Hogness, 1972). The selection and preservation of the templates in S factor and DIII may reflect such recognition.

The use of in vitro synthesizing systems as models for DNA replication is intended to provide some clues to the mechanisms of replication in vivo . These clues are often found in the nature of the components required to approach a reconstitution of the in vivo system. In the present work a protein has been studied whose addition to synthesis mixtures results in DNAs that resemble DNA produced in vivo in terms of the physical separability of the complementary strands. The unusual templates found associated with the DNA metabolizing proteins may represent isolated binding sites for these proteins. There is no evidence as to the exact in vivo significance of clc DNA, S factor, or the small templates. However, a search for such evidence provides a direction for future work in this area.

BIBLIOGRAPHY

Alberts, B. M. (1968), J. Mol. Biol. 32, 405

Alberts, B. M., and Doty, P. (1968), J. Mol. Biol. 32, 379

Barzilai, R., and Thomas, C. A. (1970), J. Mol. Biol. 51, 145

Becker, E. F., Zimmerman, B. K., and Geiduschek, E. P. (1964), J. Mol. Biol. 8, 377

Bernardi, G. (1971), Meth. Enz. 12D, 95

Boyer, H. W. (1974), Fed. Proc. 33, 1125

Bram, S. (1971), Nature New Biology 232, 174

Brutlag, D., Atkinson, M. R., Setlow, P., and Kornberg, A. (1969) Biochem. Biophys. Res. Commun. 37, 982

Brutlag, D., Sheckman, R., and Kornberg, A. (1971), Proc. Nat. Acad. Sci. USA 68, 2826

Burd, J. F., and Wells, R. D. (1970), J. Mol. Biol. 53, 435

Burgess, R. R. (1969), J. Biol. Chem. 244, 6160

Burton, K. (1967), Meth. Enz. 12A, 222

Byrd, C., Ohtsuka, E., Moon, M. W., and Khorana, H. G. (1965), Proc. Nat. Acad. Sci. USA 53, 79

Cairns, J. (1963), Cold Spring Harb. Symp. Quant. Biol. 28, 43

Cerny, R., Mushynski, W. E., and Spencer, J. H., (1968), Biochim. Biophys. Acta 169, 439

Chamberlin, M., and Berg, P. (1962), Proc. Nat. Acad. Sci. USA 48, 81

Champoux, J. J., and Hogness, D. S. (1972), J. Mol. Biol. 71, 383

Chang, L. M. S., and Bollum, F. J. (1972), Biochem. Biophys. Res. Commun. 46, 1354

Coulter, M. B., Flintoff, W. F., Paetkau, V. H., Pulleyblank, D. E., and Morgan, A. R. (1974), Biochemistry 13, 1603

Cukier-Kahn, R., Jacquet, M., and Gros, N. (1972), Proc. Nat. Acad. Sci. USA 69, 3643

Delius, H., Howe, C., and Kozinski, A. W. (1971), Proc. Nat. Acad. Sci. USA 68, 3049

DeLucia, P., and Cairns, J. (1969), Nature 224, 1164

DeWaard, A., and Lehman, I. R. (1966), in Procedures in Nucleic Acid Research, Cantoni, G. L., and Davies, D. R., Ed., New York, N.Y., Harper and Row, p 122

Englund, P. T. (1971), J. Biol. Chem. 246, 5684

Erikson, R. L., and Szybalski, W. (1964), Virology 22, 111

Flintoff, W. F. (1973), Ph.D. Thesis, University of Alberta

Flintoff, W. F., and Paetkau, V. H. (1974), Biochemistry 13, 1610

Forscheit, A. B., and Ray, D. S. (1970), Proc. Nat. Acad. Sci. USA 67, 1534

Fuchs, J. A., Karlstrom, H. O., Warner, H. R., and Reichard, P. (1972), Nature New Biology 238, 69

Fuke, M., and Inselburg, J. (1972), Proc. Nat. Acad. Sci. USA 69, 89

Gefter, M. L., Hirota, Y., Kornberg, T., Wechsler, J. A., and Barnoux, C. (1971) Proc. Nat. Acad. Sci. USA 68, 3150

Gilbert, W., Maizels, N., and Maxam, A. (1973), Cold. Spring Harbor, Symp. Quant. Biol. 38, 845

Geider, K. and Kornberg, A. (1974), J. Biol. Chem. 249, 3999

Ginsberg, B., and Hurwitz, J. (1970), J. Mol. Biol. 52, 265

Goebel, W. (1972), Nature New Biology 237, 67

Goebel, W., and Schrempf, H. (1972), Biochem. Biophys. Res. Commun. 49, 591

Goulian, M. (1968), Cold Spring Harb. Symp. Quant. Biol. 33, 11

Goulian, M. (1971), Ann. Rev. Biochem. 40 855

Guild, W. (1968), Cold Spring Harb. Symp. Quant. Biol. 33, 142

Harwood, S. J., and Wells, R. D. (1970), J. Biol. Chem. 245, 5625

Hayes, F. N., Hansbury, E., Mitchell, V. E., Ratliff, R. L., Smith, D. A., and Williams, D. L. (1971), J. Biol. Chem. 246, 3631

Heyden, B., Nusslein, C., and Schaller, H. (1972), Nature New Biology 240, 9

Hirose, S., Okazaki, R., and Tamanoi, F. (1973), J. Mol. Biol. 77, 501

Hirota, Y., Gefter, M. L., and Mindich, L. (1972a), Proc. Nat. Acad. Sci. USA 69, 3238

Hirota, Y., Mordoh, J., Scheffler, I., and Jacob, F. (1972b), Fed. Proc. 31, 1422

Ifft, J. B., Voet, D. H., and Vinograd, J. (1961), J. Phys. Chem. 65, 1138

Inman, R. B., and Baldwin, R. L. (1962), J. Mol. Biol. 5, 172

Inman, R. B., and Baldwin, R. L. (1964), J. Mol. Biol. 8, 452

Inman, R. B., and Schnos, M. (1971), J. Mol. Biol. 56, 319

Inman, R. B., Schildkraut, C. L., and Kornberg, A. (1965), J. Mol. Biol. 11, 285

Iyer, V. N., and Szybalski, W. (1964), Science 145, 55

Jacob, F., and Monod, J. (1961), J. Mol. Biol. 3, 318

Jones, O. W., and Berg, P. (1966), J. Mol. Biol 22, 199

Jovin, T. M., Englund, P. T., and Bertsch, L. L. (1969), J. Biol. Chem. 244, 2996

Kelly, T. J., and Smith, H. O. (1970), J. Mol. Biol. 51, 393

Khorana, H. G., Buchi, H., Ghosh, H., Gupta, N., Jacob, T. M., Kossel, H., Morgan, R., Narang, S. A., Ohtsuka, E., and Wells, R. D. (1966), Cold Spring Harb. Symp. Quant. Biol. 31, 39

Khorana, H. G., Jacob, T. M., Moon, M. W., Narang, S. A.,

and Ohtsuka, E. (1965), J. Amer. Chem Soc. 87, 2954

Kingsbury, D. T., and Helinski, D. R. (1973), J. Bact. 114, 1116

Kirby, K. S. (1968), Meth. Enz. 12B, 87

Knippers, R. (1970), Nature 228, 1050

Kohn, K. W., Spears, C. L., and Doty, P. (1966), J. Mol. Biol. 19, 266

Kornberg, A. (1969), Science 163, 1410

Kornberg, A., Bertsch, L. L., Jackson, J. F., and Khorana, H. G. (1964), Proc. Nat. Acad. Sci. USA 51, 315

Kornberg, T., and Gefter, M. L. (1971), Proc. Nat. Acad. Sci. USA 68, 761

Kornberg, T., and Gefter, M. L. (1972), J. Biol. Chem. 247, 5369

Kornberg, T., and Gefter, M. L. (1973), Proc. Nat. Acad. Sci. USA 70, 1764

Lane, E. A., and Mavrides, C. (1969), Anal. Biochem. 27, 363

Lark, K. G. (1972), J. Mol. Biol. 64, 47

Lehman, I. R., Bessman, M. J., Simms, E. S., and Kornberg, A. (1958), J. Biol. Chem. 233, 163

Lehman, I. R., and Chien, J. R. (1973), in DNA Synthesis in vitro , Wells, R. D., and Inman, R. B., Ed., Baltimore, University Park Press, p 3

Lehman, I. R., Roussos, G., and Pratt, E. A. (1962), J. Biol. Chem. 237, 829

Leis, J. P., and Hurwitz, J. (1972), Proc. Nat. Acad. Sci. USA 69, 2331

LePecq, J. -B., and Paoletti, C. (1967), J. Mol. Biol. 27, 87

LeTalaer, J. Y., and Jeanteur, P. (1971), Febs. Lett. 12, 253

Lin, S-Y., and Riggs, A. D. (1970), Nature 228, 1184

Magnusson, G., Pigiet, V., Winnacker E. L., Abrams, R., and Reichard, P. (1973), Proc. Nat, Acad, Sci. USA 70, 412

Maniatis, T., Ptashne, M., and Maurer, R. (1973), Cold Spring Harb, Symp. Quant. Biol. 38, 857

Masamune, Y., and Richardson, C. C. (1971), J. Biol. Chem. 246, 2692

Meselson, M., and Stahl, F. W. (1958), Proc. Nat. Acad. Sci. USA 44, 671

Meselson, M., Yuan, R., and Haywood, J. (1972), Ann. Rev. Biochem. 41, 447

Morgan, A. R. (1970), J. Mol. Biol. 52, 441

Morgan, A. R., and Paetkau, V. H. (1972), Can. J. Biochem. 50, 210

Morgan, A. R., Coulter, M. B., Flintoff, W. F., and Paetkau, V. H. (1974), Biochemistry 13, 1596

Moses, R.E. (1972), J. Biol. Chem. 247, 6031

Ohtsuka, E., Moon, M. W., and Khorana, H. G. (1965), J. Am. Chem. Soc. 87, 2956

Okazaki, R. Arisawa, M., and Sugino, A. (1971), Proc. Nat. Acad. Sci. USA 68, 2954

Okazaki, R., Okazaki, T., Sakabe, K., Sugimoto, K., and Sugino, A. (1968a), Proc. Nat. Acad. Sci. USA 59, 598

Okazaki, R., Okazaki, T., Sakabe, K., Sugimoto, K., Kainuma, R., Sugino, A., and Iwatsuki, N. (1968b), Cold Spring Harb. Symp. Quant. Biol. 33, 129

Paetkau, V. H. (1969), Nature 224, 370

Paetkau, V. H., Coulter, M. B., Flintoff, W. F., and Morgan, A. R. (1972), J. Mol. Biol. 71, 293

Paetkau, V. H., and Coy, G. R. (1972), Can. J. Biochem. 50, 142

Paetkau, V. H., and Khorana, H. G. (1971), Biochemistry 10, 1511

Painter, R. B., and Schaeffer, A. (1969), Nature 221, 1215

Pirrotta, V. (1973), Nature New Biology 244, 13

Radding, C. M., Josse, J., and Kornberg, A. (1962), J. Biol. Chem. 237, 2869

Radding, C. M., and Kornberg, A. (1962), J. Biol. Chem. 237, 2877

Richardson, C. C. (1969), Ann. Rev. Biochem. 38, 795

Richardson, C. C., Lehman, I. R., and Kornberg, A. (1964a), J. Biol. Chem. 239, 251

Richardson, C. C., Schildkraut, C. L., Aposhian, H. V., and Kornberg, A. (1964b), J. Biol. Chem. 239, 222

Rushizky, G. W., Greco, A. E., Hartely, R. W., and Sober, H. A. (1963), Biochem. Biophys. Res. Commun. 10, 311

Sadowski, P., McGeer, A., and Becker, A. (1974), Can. J. Biochem. 52, 525

Sato, S., Ariake, S., Saito, M., and Sugimura, T. (1972), Biochem. Biophys. Res. Commun. 49, 827

Schachman, H. K., Adler, J., Radding, C. M., Lehman, I. R., and Kornberg, A. (1960), J. Biol. Chem. 235, 3242

Schaller, H., Otto, B., Nusslein, V., Huf, J., Herrmann, R., and Bonhoeffer, F. (1972), J. Mol. Biol. 63, 182

Schaller, H., Voss, H., and Gucker, S. (1969), J. Mol. Biol. 44, 445

Schandl, E. K. (1972), Biochim. Biophys. Acta 262, 420

Schandl, E. K., and Taylor, J. H. (1969), Biochem. Biophys. Res. Commun. 34, 291

Schandl, E. K., and Taylor, J. H. (1971), Biochim. Biophys. Acta 228, 595

Scheffler, I. E., Elson, E. L., and Baldwin, R. L. (1968), J. Mol. Biol. 36, 291

Schildkraut, C. L., Richardson, C. C., and Kornberg, A. (1964), J. Mol. Biol. 9, 24

Schnos, M., and Inman, R. B. (1970), J. Mol. Biol. 51, 61

Schnos, M., and Inman, R. B. (1971), J. Mol. Biol. 55, 31

Sgaramella, V., van de Sande, J. H., and Khorana, H. G. (1970), Proc. Nat. Acad. Sci. USA 67, 1468

Shapiro, A. L. Vinuela, E., and Maizel, J. V. (1967), Biochem. Biophys. Res. Commun. 28, 815

Sharp, P. A., Cohen, S. N., and Davidson, N. (1973), J. Mol. Biol. 75, 235

Sigal, N. Delius, H., Kornberg, T., Gefter, M. L., and Alberts, B. (1972), Proc. Nat. Acad. Sci. USA 69, 3537

Studier, F. W. (1965), J. Mol. Biol. 11, 373

Sugino, A., Hirose, S., and Okazaki, R. (1972), Proc. Nat. Acad. Sci. USA 69, 1863

Sugino, A., and Okazaki, R. (1972), J. Mol. Biol. 64, 61

Sugino, A., and Okazaki, R. (1973), Proc. Nat. Acad. Sci. USA 70, 88

Summers, W. C., and Szybalski, W. (1968), Biochim. Biophys. Acta 166, 371

Szybalski, W., Bovre, K., Fiandt, M., Guha, A., Hradecna, Z., Kumar, S., Lozeron, H. A., Maher, V. M., Nijkamp, H. J. J., Summers, W. C., and Taylor, K. (1969), J Cell. Physiol. 74, Sup. 1, 33

Tait, R. C., and Smith, D. W. (1974), Nature 249, 116

Traub, P., and Nomura, M. (1968), Proc. Nat. Acad. Sci. USA 59, 777

Veltkamp, E., and Nijkamp, H. J. J. (1973), Mol. gen. Genet. 125, 329

Vinograd, J. (1963), Meth. Enz. 6, 854

Von Hippel, P. H., and McGhee, J. D. (1972), Ann. Rev. Biochem., 41, 231

Watson, J. D., and Crick, F. H. C. (1953), Cold Spring Harb. Symp. Quant. Biol. 18, 123

Wechsler, J. A. (1973), in DNA Synthesis in vitro , Wells, R. D., and Inman, R. B., Ed., Baltimore, University Park Press, p 375

Weigle, J., Meselson, M., and Paigen, K., (1959), J. Mol. Biol. 1, 379

Weiss, B. (1970), Proc. Nat. Acad. Sci. USA 65, 652

Weiss, B., Live, T. R., and Richardson, C. C. (1968), J. Biol. Chem. 243, 4530

Wells, R. D., and Blair, J. E. (1967), J. Mol. Biol. 27, 273

Wells, R. D., Buchi, H., Kossel, H., Ohtsuka, E., and Khorana, H. G. (1967a), J. Mol. Biol. 27, 265

Wells, R. D., Flugel, R. M., Larson, J. E., Schendel, P. F., and Sweet., R. W. (1972), Biochemistry 11, 621

Wells, R. D., Jacob, T. M., Narang, S. A., and Khorana, H. G. (1967b), J. Mol. Biol. 27, 237

Wetmur, J. G., and Davidson, N. (1968), J. Mol. Biol. 31, 349

Wells, R. D., Larson, J. E., Grant, R. C., Shortle, B. E., and Cantor, C. R. (1970), J. Mol. Biol. 54, 465

Wells, R. D., Ohtsuka, E., and Khorana, H. G. (1965), J. Mol. Biol. 14, 221

Wilson, D. A., and Thomas, C. A. (1974), J. Mol. Biol. 84, 115

Wolfson, J., and Dressler, D. (1972), Proc. Nat. Acad. Sci. USA 69, 2682